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EcR-B1 mediates the response of the Ddc gene to ecdysone in Drosophila melanogaster

by

Christian Peter Reece



A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of
the

requirements for the degree of Master of Science

in

Molecular Biology and Genetics

Department of Biological Sciences

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **EcR-B1 mediates the response of the Ddc gene to ecdysone in *Drosophila melanogaster*** submitted by **Christian Peter Reece** in partial fulfillment of the requirements for the degree of **master of science in Molecular Biology and Genetics**.

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Abstract

A pulse of the steroid hormone ecdysone initiates Pupariation in the fruit fly *Drosophila melanogaster*. The ecdysone receptor (EcR) transduces this ecdysone pulse by regulating the expression of a large number of target genes. Because there are three separate EcR isoforms (EcR-A, EcR-B1, and EcR-B2), it is generally believed that each isoform mediates the expression of a distinct set of ecdysone-inducible genes. We have shown that transcription of the *dopa decarboxylase* (*Ddc*) gene, which results in tanning and hardening of the pupal cuticle, is specifically mediated by the EcR-B1 isoform at pupariation. This evidence may largely explain how the organism manages to create tissue-specific *Ddc* transcription at pupariation, where the bulk of the expression is confined to the epidermis. In addition, we present data suggesting the cellular environment is important to the proper expression of the *Ddc* gene at the onset of metamorphosis.

Table of Contents

Introduction.....	1
Materials and methods	26
<i>Drosophila melanogaster</i> stocks	26
Collection of organisms	27
RNA isolation.....	27
Reverse transcription	28
PCR.....	29
Analysis of PCR products.....	30
Analysis of LacZ expression in larval epidermis and imaginal discs.....	30
Analysis of LacZ expression in larval extracts	31
Plasmid DNA constructs.....	32
Plasmid DNA preparation.....	32
Restriction Enzyme Digestion.....	34
Modification of DNA molecules	34
Purification of DNA molecules.....	35
Ligation of DNA molecules.....	35
Preparation of plasmid DNA for cell culture transfections	36
Maintenance of cells	36
Setup for transfection.....	37
Electroporation	37
Protein expression and ecdysone induction	38
Analysis of L57-3-11 cells transfected with the luciferase expression construct ...	39
Analysis of L57-3-11 cells with LacZ expression constructs	39
Results.....	41
The role of the EcR-B1 protein in the process of <i>Ddc</i> expression at pupariation...	41
The validity of the RT-PCR assay depends upon certain pre-established parameters.	42

The stocks carrying the <i>EcR</i> ^{Q50st} and <i>EcR</i> ^{W53st} alleles are crossed to the <i>EcR</i> ^{M554fs} null mutation to generate mutant organisms.	56
The <i>EcR-B1</i> mutations abolish <i>Ddc</i> expression at pupariation.	56
An analysis of the <i>Ddc</i> regulatory region in an <i>EcR-B1</i> mutant background	61
Stocks carrying an <i>EcR-B1</i> mutation and the relevant LacZ reporter constructs must be created prior to experimental analysis.	64
The stocks carrying the <i>EcR</i> ^{W53st} allele and the reporter constructs are crossed to the <i>EcR</i> ^{M554fs} null mutation to generate mutant organisms.	64
The EcR-B1 protein plays a role in the precocious <i>Ddc</i> expression seen in constructs lacking the silencer region.	68
The P[<i>Ddc-lacZ</i>]SH construct shows severely reduced levels of expression in an <i>EcR-B1</i> mutant background at pupariation.	73
An <i>in vitro</i> analysis of <i>Ddc</i> regulation in a cell culture system	73
The L57-3-11 cell line responds to ecdysone following transfection.	77
The <i>Ddc-lacZ</i> constructs are not induced when co-transfected with the EcR expression vectors.	77
The construction of vectors that express the BR-C protein isoforms.	84
The <i>Ddc-lacZ</i> constructs are not induced when co-transfected with the EcR and BR-C expression vectors.	96
Discussion	106
The role of EcR-B1 in <i>Ddc</i> induction at pupariation.	107
EcR-B1 and the silencing element(s) of the <i>Ddc</i> promoter	109
Analysis of <i>Ddc</i> expression in a cell-culture system	111
Conclusions	113
Bibliography	118

List of Tables

Table II-1: Plasmid Constructs.....	33
-------------------------------------	----

List of Figures

Figure I-1: DDC activity and ecdysone titre during the life cycle of <i>Drosophila melanogaster</i>	4
Figure I-2: The Ashburner model.....	8
Figure I-3: The domain structure of nuclear receptors.....	17
Figure III-1: Determining the linearity of input RNA from sample 11	43
Figure III-2: Determining the linearity of input RNA from sample 12	45
Figure III-3: Determining the linearity of input RNA from sample 13	47
Figure III-4: Determining the linearity of PCR reaction from sample 11	50
Figure III-5: Determining the linearity of PCR reaction from sample 12	52
Figure III-6: Determining the linearity of PCR reaction from sample 13	54
Figure III-7: Generation of <i>EcR-B1</i> mutant organisms.....	57
Figure III-8: Comparative RT-PCR analysis of <i>Ddc</i> expression in <i>EcR-B1</i> and wild-type organisms at pupariation	59
Figure III-9: Diagram of the <i>Ddc-lacZ</i> constructs used in this thesis	62
Figure III-10: Diagram of the crossing scheme used to combine the <i>EcR</i> ^{W53st} mutant with the P[<i>Ddc-lacZ</i>] reporter constructs.	65

Figure III-11: Generation of <i>EcR-B1</i> mutant organisms that carry the P[<i>Ddc-lacZ</i>]SH, P[<i>Ddc-lacZ</i>]BH, and P[<i>Ddc-lacZ</i>]RH constructs	69
Figure III-12: Expression patterns of the P[<i>Ddc-lacZ</i>] constructs in the epidermis of <i>EcR</i> ^{W53st} and wild-type wandering third instar larvae.....	71
Figure III-13: Activity of the P[<i>Ddc-lacZ</i>]SH construct in <i>EcR</i> ^{W53st} / <i>EcR</i> ^{M554fs} and wild-type organisms.....	74
Figure III-14: β -galactosidase activity in L57-3-11 cells transfected with the P[<i>Ddc-lacZ</i>]SH construct	79
Figure III-15: β -galactosidase activity in L57-3-11 cells transfected with the P[<i>Ddc-lacZ</i>]BH construct.....	82
Figure III-16: β -galactosidase activity in L57-3-11 cells transfected with the P[<i>Ddc-lacZ</i>]RH construct.....	85
Figure III-17: Schematic diagram of the BR-C expression vectors.....	87
Figure III-18: Analysis of the BR-C expression vectors by restriction enzyme digestion.....	91
Figure III-19: PCR analysis of the BR-C expression constructs	94
Figure III-20: The effects of exogenous BR-C Z2 protein on P[<i>Ddc-lacZ</i>]SH-generated β -galactosidase activity in L57-3-11 cells.....	98
Figure III-21: The effects of exogenous BR-C Z1 protein on P[<i>Ddc-lacZ</i>]SH-generated β -galactosidase activity in L57-3-11 cells.....	100

Figure III-22: The effects of exogenous BR-C Z3 protein on
P[Ddc-lacZ]SH-generated β -galactosidase activity in L57-3-11
cells 102

Figure III-23: The effects of exogenous BR-C Z4 protein on
P[Ddc-lacZ]SH-generated β -galactosidase activity in L57-3-11
cells 104

Introduction

The Earth is populated by an incredible array of complex and highly specialized multicellular organisms. Without exception, these organisms begin their existence as single-cell zygotes and only achieve their final form through a complicated process involving cell division, cell differentiation, and programmed cell death. This process is governed by a wide variety of developmental signals that must be faithfully converted into appropriate levels of spatially and temporally restricted gene expression if the organism is to achieve its correct mature form. Failure to generate the appropriate levels of gene expression at the correct times and places within an organism will typically result in some degree of deformity or even death.

Today, we know of many different categories of these developmental signals. Of these, one of the most intensively studied categories is mediated by a class of chemicals known as hormones. Hormones are intercellular chemical signals that are secreted from a source tissue and dispersed through the blood or haemolymph in a fashion that results in physiologically active concentrations throughout the organism. However, the systemic presence of any one hormone tends to produce a disparate array of responses amongst the various target tissues in the organism. Within the framework of development, a hormone will often cause certain cell types to begin replicating; others may begin to further differentiate; and yet still others may respond by initiating the process of programmed cell death. Furthermore, many cell types will undergo a combination of the aforementioned processes and yet others will fail to

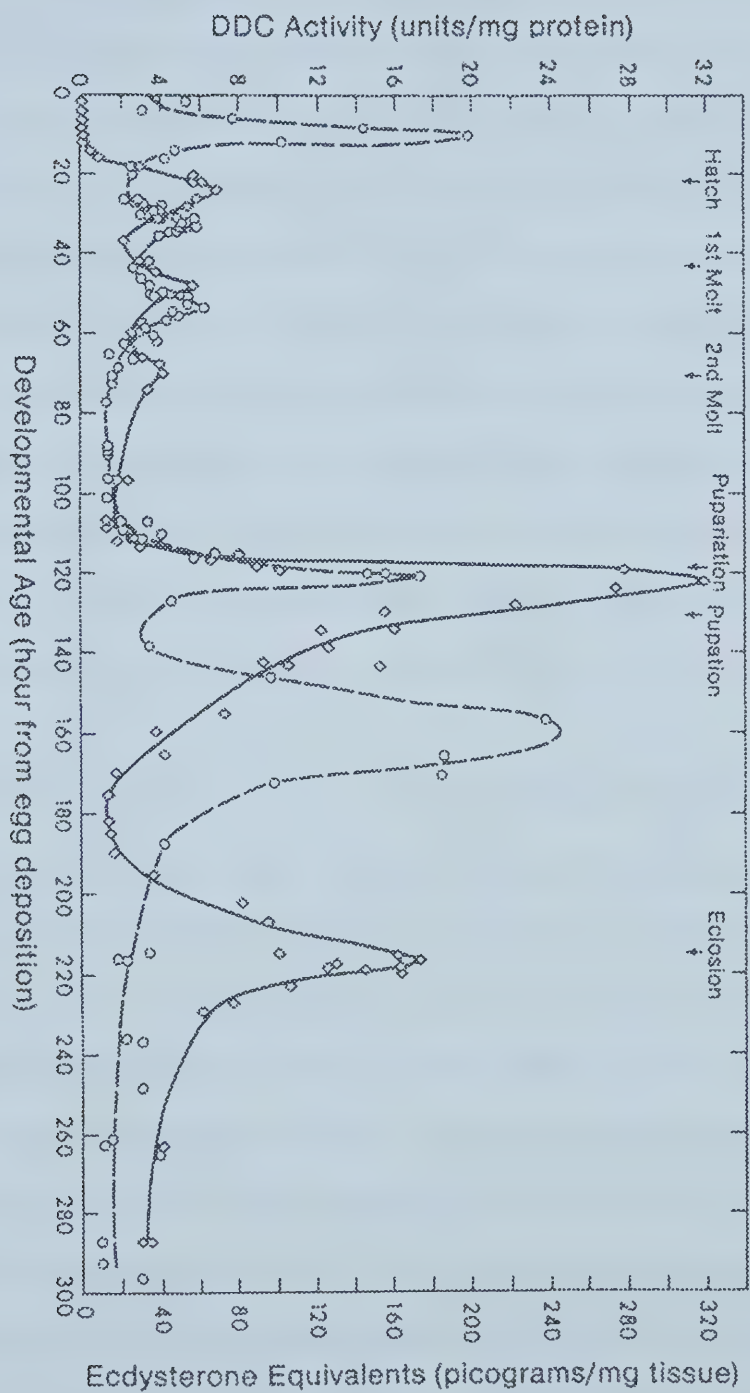
respond to a hormonal signal in any fashion. This suggests that the state of a given tissue, referred to as its competency, plays a large role in mediating that tissue's response to a hormonal signal. Understanding how different tissues obtain their unique forms of competency is the subject of much present day research.

Our knowledge of the molecular mechanisms that underlie the responses to developmental signals is based, in large part, upon studies done in model organisms that are amenable to genetic analysis. The use of one such model organism, *Drosophila melanogaster*, has been especially helpful in our attempts to understand how hormones control gene expression. A dipteran insect, the life cycle of *Drosophila melanogaster* consists of seven stages: embryonic, three larval instars, prepupal, pupal, and adult. It is the steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone) that controls these transitions from one stage to the next in the organism (reviewed in Riddiford, 1993). Closely linked with each transition from one stage to the next is the need to harden and tan the outer cuticular layer of the organism. This process, known as sclerotization, has been associated with the release of ecdysone from the time of the pioneering work of Fraenkel (1935). It is now known that the enzyme Dopa decarboxylase (DDC) controls the synthesis of the chemicals which ultimately sclerotize the cuticle (reviewed in Wright, 1987). This link has made the *dopa decarboxylase* (*Ddc*) gene an excellent model for studying ecdysone-induced gene expression in *Drosophila melanogaster*.

The *Ddc* gene has been cloned by Hirsh and Davidson (1981) and has been shown to be a single structural gene controlled by a single promoter. Furthermore, work by Scholnick *et al.* (1983) has shown that a single 7.5 kb *Pst* I restriction fragment that encompasses the exons of the *Ddc* gene is capable of restoring wild-type function to *Ddc* mutant organisms upon P-element-mediated transformation. Despite the relatively short and simple structure of the *Ddc* gene, the expression patterns of the gene product are quite complex. Within the cells of the central nervous system (CNS), DDC is required to generate the neurotransmitters dopamine and serotonin (Wright, 1987). Consistent with the continuous requirement for neurotransmitters, expression levels within the CNS are quite constant, but account for a relatively small amount of overall DDC activity (Scholnick *et al.*, 1986). On the other hand, the cells of the epidermis require DDC to generate dopamine, which is then converted into N-acetyl-dopamine quinone and N- β -alanyldopamine quinone, which effect sclerotization of the cuticle (Wright, 1987). In accordance with the periodic need for cuticle sclerotization, epidermal DDC levels fluctuate a great deal and peak titres account for over 90% of total DDC activity in the organism (Marsh and Wright, 1980; Kraminsky *et al.*, 1980; Figure I-1).

To gain a better understanding of the relationship between the levels of ecdysone and the expression of DDC in the epidermis of *Drosophila*, Kraminsky *et al.* (1980) performed a comparative analysis of the levels of the two molecules throughout the development of the organism (Figure I-1). Their results showed that four of the five

Figure I-1 DDC activity and ecdysone titre during the life cycle of *Drosophila melanogaster*. The DDC activity ($\diamond$) and the ecdysone titre ($\circ$) were plotted as a function of the age in hours from egg deposition of the Canton-S wild type strain when reared at 25°C. This figure is a reproduction of the one originally found in Kraminsky *et al.* (1980).



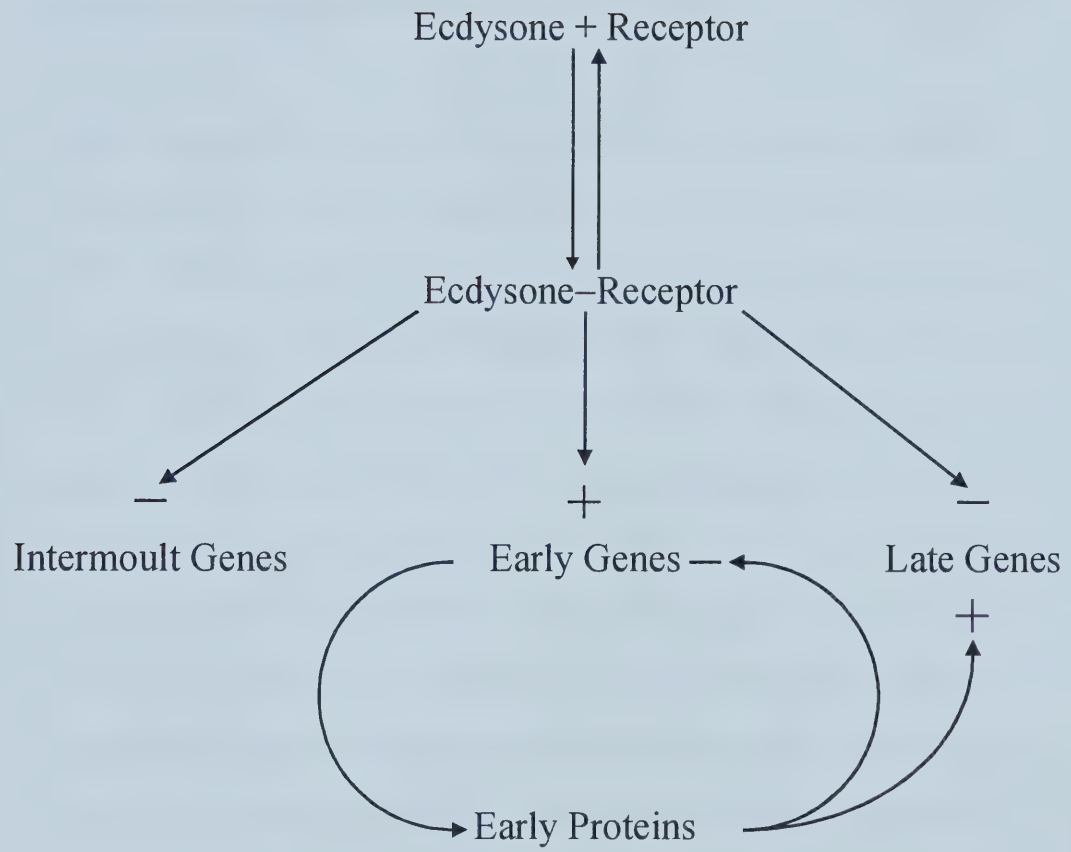
peaks of DDC activity lag behind their associated peaks of ecdysone expression, while only the peak of DDC activity at pupariation was coincident with a peak in the ecdysone titre. This suggested that DDC expression in the epidermis was under at least two distinct forms of control, with only the peak of expression at pupariation directly induced by an increasing titre of ecdysone. To confirm the latter hypothesis, Kraminsky and co-workers made use of the *ecd^l* mutant strain of *Drosophila melanogaster* (Garen *et al.*, 1977), which fails to produce ecdysone when kept at 29°C. When late third instar *ecd^l* larvae were placed at the restrictive temperature, it was shown that these organisms were incapable of pupariating and failed to produce translatable *Ddc* mRNA. However, exogenous administration of ecdysone caused a rapid increase in both translatable *Ddc* mRNA and DDC activity, demonstrating that an increase in the titre of the hormone results in DDC expression at pupariation.

The results of Kraminsky *et al.* are consistent with those of Ashburner *et al.* (1974). In an elegant set of experiments, Michael Ashburner and his colleagues studied the response of explanted salivary glands to treatment with ecdysone by monitoring the puffing patterns that occur in the giant polytene chromosomes present in the tissue. When the polytene chromosomes first become apparent in the middle portion of the third instar stage, they display a number of puffs at characteristic points along the chromosomes. Ashburner and colleagues used the term “intermoult puffs” to describe these features of third instar salivary gland polytene chromosomes. They found that upon addition of ecdysone, the intermoult puffs disappeared and a small number of what they termed “early puffs” were generated within minutes.

Furthermore, in the continued presence of ecdysone, these puffs would reach a maximum size after about four hours and then regress. They also found that after a lag period of approximately three hours, a much larger set of so-called “late puffs” appeared. In addition, the early withdrawal of ecdysone resulted in the premature appearance of these late puffs. Finally, a repetition of these experiments in the presence of the protein synthesis inhibitor, cycloheximide, produced a number of interesting results. The researchers found that the appearance of the early puffs was entirely independent of protein synthesis at that point in time. They also found that the appearance of the late puffs was entirely dependent upon protein synthesis at that time. Finally, they found that in the continuous presence of ecdysone, the regression of the early puffs was dependent on protein synthesis.

Based on the aforementioned results, Ashburner and colleagues generated a model for hormone action in salivary glands at pupariation that largely holds true to this day (Ashburner *et al.*, 1974; Figure I-2). The so-called Ashburner model, based on work showing that these puffs represent transcriptionally active genes (Beermann, 1961; Lambert, 1972; Lambert and Daneholt, 1975), assumed that the intermoult puffs represented active transcription of the “intermoult genes”, the early puffs represented active transcription from “early genes”, and the late puffs represented active transcription from “late genes”. The model proposed that ecdysone, upon binding its pre-synthesized receptor protein, had two opposing regulatory effects: it directly induced transcription from the early genes and it directly repressed transcription from

Figure I-2 A schematic representation of the Ashburner model of the ecdysone-induced regulatory hierarchy of gene expression that underlies the puffing pattern of the polytene chromosomes at pupariation (Ashburner *et al.*, 1974). This figure is an adaptation of the one that originally appeared in Andres and Thummel (1992).



the intermoult and late genes. Next, it proposed that the protein products of the early genes acted to induce transcription of the late genes. Finally, the model proposed that the early genes function as autoregulators, repressing their own transcription and thus attenuating the response to ecdysone.

Using the Ashburner model as a framework, the *Ddc* gene was initially thought to behave much like a classic early gene based upon the work of Kraminsky *et al.* (1980). However, later work by Clark *et al.* (1986) brought this view into question. They found that upon exogenous administration of both ecdysone and cycloheximide to mature *ecd^l* larvae maintained at 29°C, *Ddc* transcript levels increased 6-fold compared to untreated *ecd^l* larvae. However, when this experiment was conducted in the absence of cycloheximide, it generated a 25-fold increase in *Ddc* transcript levels when compared to untreated *ecd^l* larvae. In fact, these results were consistent with the already published findings of Ashburner and Richards (1976). Based on their own evidence, Ashburner and Richards proposed subdividing the late genes into an “early-late” class and a “late-late” class. The category they described as early-late genes showed a decrease, but not a total loss, of ecdysone-induced activity in the presence of protein synthesis inhibitors. On the other hand, the category they described as late-late genes were characterized by a complete loss of ecdysone-induced activity in the presence of protein synthesis inhibitors. The results of Clark *et al.* (1986) indicate that the *Ddc* gene behaves like an early-late gene at pupariation, with a requirement for a direct input from the ecdysone-receptor complex and a

requirement for an input from one or more early gene proteins to produce maximal induction.

Our understanding of how the early gene proteins may regulate *Ddc* transcription at pupariation has been greatly aided by the molecular characterization of the 2B5 (DiBello *et al.*, 1991), 74EF (Segraves and Hogness, 1990), and 75B (Burtis *et al.*, 1990) early puff loci, which are now known as the *Broad-Complex (BR-C)*, *E74*, and *E75* genes, respectively. All three genes are induced by ecdysone as a primary response, all three genes have been shown to encode transcription factors, and, in the cases of *E74* (Urness and Thummel, 1990) and *E75* (Hill *et al.*, 1993), been shown to bind to early and late puff loci. These results are all consistent with the properties of early genes proposed by the Ashburner model. Furthermore, studies have shown that these three early genes are expressed in a variety of tissues at the onset of metamorphosis (Thummel *et al.*, 1990; Huet *et al.*, 1993; Emery *et al.*, 1994) and that mutations in the *BR-C* and *E74* genes have widespread effects on the development of larval and imaginal tissues (Kiss *et al.*, 1988; Fletcher *et al.*, 1995). Finally, all three genes are unusually large (60 to 100 kb) and complex, with multiple promoters that give rise to different processed mRNAs. Taken together, these results support an extension of the Ashburner model called the tissue co-ordination model (Thummel *et al.*, 1990; Burtis *et al.*, 1990). The model proposed that different target tissues respond to ecdysone in a fashion similar to the way proposed by Ashburner *et al.* (1974) for the larval salivary gland. Furthermore, it proposed that the differential nature of the response in each target tissue at metamorphosis was largely the result of

the expression of different but overlapping combinations of the transcription factors encoded by the early genes.

One of these early genes, the *Broad-Complex* (*BR-C*), has been shown to be required to generate wild-type levels of DDC activity at pupariation (O’Keefe *et al.*, 1995; Hodgetts *et al.*, 1995). Furthermore, the nature of this relationship has been defined on the basis of the large number of extant *BR-C* alleles. These mutant alleles have been placed into four complex complementation groups based on the work of Kiss *et al.* (1988). The first three complementation groups, *broad* (*br*), *reduced bristles on the palpus* (*rbp*), and *2Bc*, represent three genetic subfunctions and fully complement each other. The fourth group, termed *nonpupariating* (*npr1*), fails to complement any of the other three groups. By analyzing both the *Ddc* transcript levels (O’Keefe *et al.*, 1995) and DDC activity levels (Hodgetts *et al.*, 1995) in various *BR-C* mutants, it has been shown that the *br* function is necessary for full DDC activity at pupariation. Furthermore, neither the *rbp* function nor the *2Bc* function seems to have a significant effect on *Ddc* mRNA levels or DDC activity.

The molecular structure of the *BR-C* locus is also very complex, giving rise to a large family of RNA transcripts that encode several protein isoforms which are all related to one another by a common “core” region (Bayer *et al.*, 1996; Karim *et al.*, 1993; Karim and Thummel, 1992; DiBello *et al.*, 1991). The various isoforms are generated by producing transcripts with the exons encoding the core region alternatively spliced to one of four exons, termed Z1, Z2, Z3, and Z4, which encode unique pairs of zinc-finger motifs, located at the carboxy-terminal end of the proteins.

Although the relationship between these isoforms and the genetic complementation groups is complicated, there is strong evidence to suggest that the BR-C Z2 isoform is responsible for the *br*⁺ function. This was first suggested by a molecular analysis of the *br*²⁸ allele, which showed that this mutation was caused by a P-element insertion in the exon encoding the Z2-specific zinc finger motif (DiBello *et al.*, 1991; Schouls, 1993; Emery *et al.*, 1994). Subsequently, Bayer *et al.* (1997) demonstrated that the lethality associated with *br* null alleles could be rescued by heat-inducible expression of a BR-C Z2 transgene. They also demonstrated that heat-induced transgenic expression of the Z1, Z3, and Z4 isoforms failed to rescue the *br* alleles.

Furthermore, Bayer and her colleagues showed that induction of the Z2 transgene in late-third instar *br*⁵ larvae rescued *Ddc* transcript levels, whereas expression of the Z1, Z3, or Z4 transgenes failed to rescue *Ddc* transcript levels. Combined with the evidence of reduced *Ddc* transcript levels and DDC activity in *br* mutations, it is now firmly believed that full induction of the *Ddc* gene in the epidermis at pupariation requires expression of the BR-C Z2 protein.

Although it is tempting to assume that expression of a single BR-C isoform produces tissue-specific gene expression, the evidence suggests this is not the case. For example, analyses of the fat body (Mugat *et al.*, 2000), ovaries (Tzolovsky *et al.*, 1999), and larval epidermis (Hodgetts *et al.*, 1995) indicate that concurrent expression of all four BR-C isoforms takes place in these tissues. Instead, the recent work of Mugat *et al.* (2000) suggests that dynamic expression levels of BR-C isoforms are responsible for tissue-specific gene expression at the onset of metamorphosis. Their

research focused on a reporter construct that under normal conditions is only expressed in the fat body during the second half of the third larval instar stage. They found that by prematurely expressing the BR-C Z3 isoform during the first half of the third larval instar, when only the Z2 isoform is normally present in the fat body, they generated precocious expression of the reporter construct. Conversely, overexpression of the BR-C Z2 isoform in the latter stages of the third larval instar, when Z3 levels are high and Z2 levels are normally very low in the fat body, resulted in repression of the reporter construct when it is normally active. Recent evidence suggests that epidermal-specific *Ddc* expression at pupariation is under a similar form of control, with the gene being kept in an inactive state until high relative levels of the BR-C Z2 isoform are generated by the late-third instar ecdysone pulse (Chen *et al.*, 2002b).

Compared with our knowledge of how the *BR-C* products control *Ddc* expression, we have a relatively poor understanding of the role the ecdysone-receptor complex plays in directly inducing the *Ddc* gene at pupariation. However, a surprisingly large amount of data have been accumulated in recent years relating to the structure and function of the ecdysone receptor. The *ecdysone receptor (EcR)* gene has been cloned by Koelle *et al.* (1991). They demonstrated that the corresponding EcR protein could bind to ecdysone and that it was capable of activating transcription from the previously identified ecdysone response element (EcRE) (Riddihough and Pelham, 1987) in a hormone-dependent manner. Furthermore, their analysis of the *EcR* DNA sequence indicated that the predicted protein was a member of the nuclear

receptor superfamily (reviewed in Mangelsdorf *et al.*, 1995), possessing the requisite DNA-binding domain (DBD) and ligand-binding domain (LBD), with an amino acid sequence that shared a very high degree of similarity with the vertebrate retinoic acid receptor (RAR). Subsequently, it was shown that the mature receptor with high affinity ecdysone-binding and DNA-binding activities was actually a heterodimer consisting of the EcR protein and the Ultraspiracle (USP) protein, a product of the *ultraspiracle (usp)* gene (Yao *et al.*, 1992; Yao *et al.*, 1993). Interestingly, the vertebrate homolog of Usp, the retinoid X receptor (RXR) (Oro *et al.*, 1990; Mangelsdorf *et al.*, 1990), has been shown to dimerize with the RAR protein (reviewed in Mangelsdorf and Evans, 1995). This evolutionary conservation also extends to the functional level, with Usp and RXR being able to functionally substitute for each other (Yao *et al.*, 1992; Thomas *et al.*, 1993).

It is now known that the *EcR* gene encodes three functional protein isoforms, termed EcR-A, EcR-B1, and EcR-B2 (Talbot *et al.*, 1993). The transcripts for both the EcR-B1 and EcR-B2 proteins are generated from a single transcription unit driven by a single promoter. A primary transcript is produced which is then alternatively spliced to generate the mature transcripts, with the *EcR-B1* mRNA containing exons 1 through 6 and the *EcR-B2* mRNA containing exon 1 spliced to exons 3 through 6. The translation initiation codon of the *EcR-B2* open reading frame (ORF) is encoded in exon 1 while a stop codon in the 5' region of exon 2 leads to the *EcR-B1* ORF beginning at a translation initiation codon in exon 2. A second, overlapping transcription unit with a distinct promoter is responsible for generating the *EcR-A*

transcript. The *EcR-A* mRNA consists of exons A1 through A3 fused to the same exons 3 through 6 of the B isoform transcripts. The *EcR-A* ORF begins at a translation initiation codon in exon A2. Translation of the three mRNAs generates three protein isoforms that have distinct amino-terminal sequences fused to a common 652 residue C-terminal region. Finally, all three isoforms must dimerize with Usp to form functional receptors.

With some rare exceptions, the nuclear receptors show an identical structural organization consisting of an amino-terminal A/B region, followed by the DBD (C region), a linker region (D region), the LBD (E region), and a carboxy-terminal F region with no known function (Figure I-3; Moras and Gronemeyer, 1998; Mangelsdorf *et al.*, 1995). As shown in Figure I-3, all three EcR isoforms conform to this structural organization, with the unique amino-terminal domain of each isoform corresponding to the A/B region and the common carboxy-terminal domain of all three isoforms corresponding to the C through F regions (Talbot *et al.*, 1993; Koelle *et al.*, 1991). In addition, vertebrate nuclear receptors have been shown to possess two autonomous transactivation domains that are responsible for the receptors' ability to induce transcriptional activity (Gronemeyer and Laudet, 1995). The first transactivation domain, termed activation function 1 (AF-1), resides in the A/B region and functions in a ligand-independent fashion. The second transactivation domain, termed activation function 2 (AF-2), resides in the LBD and functions in a ligand-dependent fashion. Therefore, it seems likely that each EcR isoform should possess a

Figure I-3 The domain structure of nuclear receptors. (A) The five conserved regions of the nuclear receptor superfamily described in the text are shown superimposed over a generic nuclear receptor molecule. The DNA-binding domain (DBD) is contained in the C region and the Ligand-binding domain (LBD) is contained in the E region. (B) The structural organization of EcR-B1, EcR-B2, and EcR-A (denoted as B1, B2, and A, respectively) are shown in comparison to each other and the generic nuclear receptor molecule. The three EcR isoforms are identical except in the A/B regions that are entirely unrelated. N, amino-terminal end of the protein. C, carboxy-terminal end of the protein.

A)



B)



unique AF-1 and all three EcR isoforms should possess a common AF-2. Recently, Hu *et al.* (2003) and Cherbas *et al.* (2003) have provided evidence that all three EcR isoforms do possess both an AF-1 and an AF-2. Furthermore, Hu *et al.* (2003) have shown that the A/B regions of the three EcR isoforms differ in their ability to activate transcription of a semisynthetic promoter, consistent with each isoform possessing a unique AF-1.

The determination of the three-dimensional structures of a number of nuclear receptor LBDs, including LBDs in the unliganded (apo-), agonist-bound (holo-), and antagonist-bound states, has been invaluable to our understanding of the mechanism by which nuclear receptors regulate the transcriptional activity of their target genes. The crystal structures of all nuclear receptor LBDs studied to date (Yamada *et al.*, 2003) have been shown to conform to the common fold described by Wurtz *et al.* (1996). This common fold consists of 12 α -helices (numbered H1–H12) and one β -turn arranged into a three-layer “sandwich” that forms the ligand-binding pocket. When the LBD is in its unliganded state, it takes on a final conformation with H12, the α -helix closest to the carboxy-terminal end of the protein, projecting away from the LBD. However, the crystal structures of the holo-LBDs indicate that a number of structural alterations take place upon ligand binding. Most notably, H12 folds over and forms a “lid” on the ligand-binding pocket, resulting in the formation of the AF-2 surface. The importance of H12 to the formation of AF-2 is further emphasized by the crystal structures of antagonist-bound LBDs, which show that the antagonists function by preventing the transconformation of this α -helix (Brzozowski *et al.*,

1997; Shiau *et al.*, 1998; Pike *et al.*, 1999). When these nuclear receptors are in an unliganded state, they often interact with a co-repressor complex that exhibits a histone deacetylase activity (Torchia *et al.*, 1998). Upon binding to their ligands, the nuclear receptors are believed to dissociate from this co-repressor complex as a result of the change in the conformation of their LBDs. The ligand-bound nuclear receptors, through their newly formed AF-2s, then become capable of interacting with a co-activator complex that possesses a histone acetyltransferase activity (Torchia *et al.*, 1998). Previous studies have shown that histone hypoacetylation is associated with transcriptionally silent chromosomal domains, while acetylation of the lysine residues of certain histones is associated with increased transcription (Grunstein, 1997; Wolffe *et al.*, 1997). Thus, it is felt that the transcriptional repression mediated by apo-nuclear receptors and the transcriptional activation mediated by holo-nuclear receptors is due to these histone deacetylase and histone acetyltransferase activities, respectively.

Although the three-dimensional structure of the LBD of the three EcR isoforms has yet to be determined, a number of lines of evidence suggests that it likely functions in much the same way as the aforementioned vertebrate nuclear receptors. First, the similarity of the amino acid sequence of the EcR LBD (Talbot *et al.*, 1993, Koelle *et al.*, 1991) to the amino acid sequences of nuclear receptors with known crystal structures suggests that it also assumes the common fold described by Wurtz *et al.* (1996). Second, certain alterations of the highly conserved amino acids in the presumptive H12 of the EcR LBD generate receptor molecules that act in a dominant-

negative manner (Cherbas *et al.*, 2003). Third, a nuclear receptor co-repressor, SMRTER (Tsai *et al.*, 1999), and three potential nuclear receptor co-activators, TAIMAN (Bai *et al.*, 2000), the *nej* protein product (Akimaru *et al.*, 1997), and BONUS (Beckstead *et al.*, 2001), have been identified in *Drosophila melanogaster*.

In general, much less is known about the AF-1 of nuclear receptors. However, research done directly in *Drosophila* has shed some light on this aspect of EcR function. Specifically, the generation of antibodies to the EcR-A and EcR-B1 protein isoforms allowed Talbot *et al.* (1993) to determine the expression patterns of these two proteins at the onset of metamorphosis. The authors demonstrated that a roughly complementary pattern of expression exists at this stage. Essentially, tissues that stained strongly with antibodies directed against the one EcR isoform typically stained weakly with antibodies directed against the other EcR isoform. Furthermore, the EcR isoform that predominated in any one tissue was found to correlate well with the fate of that tissue at the onset of metamorphosis. Specifically, the imaginal discs, which give rise to adult structures, were shown to uniformly exhibit a very high EcR-A to EcR-B1 ratio. On the other hand, the larval tissues, which are fated to die by histolysis, were shown to have a very high EcR-B1 to EcR-A ratio. These results suggest that the various EcR isoforms play a role in generating the different responses of the various tissues at metamorphosis. However, the absence of an anti-EcR-B2 antibody makes it impossible to demonstrate what tissues, if any, express this isoform at the onset of metamorphosis. This, in turn, somewhat weakens the argument that the differential expression of EcR isoforms helps to determine metamorphic fate.

Specifically, it is possible that the EcR-B2 receptor isoform mediates both the ecdysone-dependent death of larval tissues and the ecdysone-dependent survival and proliferation of the imaginal discs.

Further evidence in support of this hypothesis has come from the isolation and characterization of mutations that specifically interrupt EcR-B1 function (Bender *et al.*, 1997). The authors have demonstrated that these *EcR-B1* mutants survive until the beginning of pupariation, suggesting that EcR-B1 function is dispensable during the embryonic and larval stages of life. However, at the point where the larval to prepupal transition normally occurs, it was shown that a number of EcR-B1-predominant tissues failed to initiate their expected metamorphic response to the late-larval pulse of ecdysone. In contrast, the leg imaginal discs, where the EcR-A isoform predominates, showed definite signs of elongation, consistent with their metamorphic response in wild-type organisms. Furthermore, an analysis of the polytene chromosomes of the salivary glands, an EcR-B1-predominant tissue, found that puffing at a number of early and early-late gene loci was severely reduced. The authors were capable of regenerating a near wild-type level of puffing in the *EcR-B1* mutant by expressing EcR-B1 protein from a transgene under heat shock control. On the other hand, expression of EcR-B2 from a transgene resulted in only a partial restoration of the puffing pattern and expression of EcR-A from a transgene resulted in little noticeable puffing.

Although the tissue co-ordination model (Thummel *et al.*, 1990; Burtis *et al.*, 1990) proposed that different but overlapping sets of transcription factors were responsible for at least a portion of each tissue's specific response to the prepupal ecdysone pulse, it didn't attempt to answer how these differences are generated in the first place. Based on the aforementioned results, it would appear that the various EcR isoforms play a role in generating these different sets of transcription factors in ecdysone target tissues. Interestingly, the epidermis of late-third larval instar organisms, which is the site of the majority of DDC protein synthesis, predominantly expresses the EcR-B1 protein isoform (Talbot *et al.*, 1993). Therefore, it is quite possible that EcR-B1 protein mediates the induction of the *Ddc* gene at pupariation. Furthermore, because the ecdysone-receptor complex drives expression at the *BR-C* locus, it is possible that EcR-B1 protein exerts this control over the *Ddc* gene by driving expression of the BR-C Z2 isoform. On the other hand, it is possible that EcR-B1 controls *Ddc* expression by binding to the gene and recruiting the transcription factors, possibly including BR-C Z2, that are necessary to generate high levels of transcriptional activity at pupariation. Most likely, the induction of the *Ddc* gene at pupariation is explained by some combination of these two possibilities.

Lastly, although the tissue coordination model has been largely supported by subsequent experimental evidence, it provides a somewhat simplistic explanation of the mechanism by which ecdysone induces the process of pupariation. Transcripts from the *BR-C*, *EcR*, and *E74* early genes can be detected more than a day before pupariation occurs (Huet *et al.*, 1993; Andres *et al.*, 1993). Furthermore, the work of

Andres *et al.* (1993) indicates that a low titre pulse of ecdysone in early-third instar larvae is responsible for this transcription. This added complexity raises questions in regards to *Ddc* gene expression within the context of the whole third instar stage of development. The work of Andres *et al.* does show that *Ddc* expression is limited to a six to eight hour window just prior to pupariation, in agreement with the work of Kraminsky *et al.* (1980). However, does the early-third instar ecdysone pulse fail to induce *Ddc* transcription because it does not reach a critical threshold concentration? Or is the expression of *Ddc* somehow actively repressed in early-third instar larvae? And possibly most important, does this pulse of ecdysone in early-third instar larvae play a role in generating the EcR-B1 protein isoform? The answers to these questions are fundamental to our understanding of how ecdysone regulates the expression of the *Ddc* gene.

The overarching goal of this thesis is to provide a more thorough understanding of how the ecdysone receptor mediates expression of the *Ddc* gene. More specifically, this study will attempt to define and expand upon what is presently a potential role for the EcR-B1 protein in mediating the induction of *Ddc* gene expression at pupariation by ecdysone. First, this thesis will attempt to demonstrate conclusively that the EcR-B1 participates in the induction of *Ddc* at pupariation. Then, based on these results, this study will look at how the EcR-B1 protein functions in relation to newly defined *Ddc* regulatory regions that contain a suppressor element(s), a *BR-C*-interacting element(s), and an EcRE (Chen *et al.*, 2002a; Chen *et al.*, 2002b). Lastly, this thesis will work to develop a cell-culture system that will allow for an *in vitro* analysis of

ecdysone-induced *Ddc* expression. The hope is that this system will provide an expedient mechanism for identifying and defining regulatory elements that are critical for proper ecdysone-induced expression of the *Ddc* gene.

Materials and methods

Drosophila melanogaster stocks

Details and precise phenotypes of the genes listed below can be found in Lindsley and Zimm (1992). The following stocks were used in this study:

yw; EcR^{W53st} / CyO, y⁺ — A stock carrying an *EcR* mutation that generates a stop codon in the *EcR-B1*-specific exon, resulting in an EcR-B1 protein truncated after only 52 amino acid residues (Bender *et al.*, 1997).

yw; EcR^{Q50st} / CyO, y⁺ — A stock carrying an *EcR* mutation that generates a stop codon in the *EcR-B1*-specific exon, resulting in an EcR-B1 protein truncated after only 49 amino acid residues (Bender *et al.*, 1997).

yw; EcR^{M554fs} / CyO, y⁺ — A stock carrying an *EcRM^{554fs}*, a null mutation of the gene (Bender *et al.*, 1997).

P[Ddc-lacZ]SH / P[Ddc-lacZ]SH — A stock carrying a third chromosome insert of a *Ddc* promoter-*lacZ* reporter fusion that possesses the putative EcRE, *BR-C*-interacting element(s), and silencing element(s) (Chen *et al.*, 2002a; Chen *et al.*, 2002b).

P[Ddc-lacZ]BH / P[Ddc-lacZ]BH — A stock carrying a third chromosome insert of a *Ddc* promoter-*lacZ* reporter fusion that possesses the putative EcRE and *BR-C*-interacting element(s) (Chen *et al.*, 2002a; Chen *et al.*, 2002b).

P[Ddc-lacZ]RH / P[Ddc-lacZ]RH — A stock carrying a third chromosome insert of a *Ddc* promoter-*lacZ* reporter fusion that possesses the putative EcRE (Chen *et al.*, 2002a; Chen *et al.*, 2002b).

Roi / Sp ; Sb / Ly — A stock carrying a second chromosome balancer chromosome marked with *Roi*, a second chromosome with the dominant marker *Sp*, a third chromosome balancer chromosome marked with *Sb*, and a third chromosome with the dominant marker *Ly* (gift of John Bell).

Oregon R — A common wild-type laboratory stock.

Collection of organisms

In all cases, stocks of *Drosophila melanogaster* carrying the *EcR-B1*-specific mutations were crossed to a stock carrying the *EcR*^{M554fs} null mutation to generate organisms with the lethal genotype. Third instar larvae were staged and collected as described in Andres and Thummel (1994), using the “blue gut” technique. The phenotype of each organism was classified as *yellow*[−] or *yellow*⁺ based on the pigmentation of the mouthparts and denticle belts. Each organism was then flash frozen in liquid nitrogen and stored at −70°C.

RNA isolation

Larvae were placed in a 1.5 ml microcentrifuge tube and homogenized with a plastic micropestle in the presence of TRIzol reagent (Invitrogen). Isolation of RNA was done according to the protocol supplied by the manufacturer. Mass isolation of OrR RNA was done using 25 late third instar larvae and 1.0 ml of the TRIzol reagent. The RNA from each mutant and sibling strain was isolated using 6 organisms of the relevant genotype and 0.8 ml of TRIzol Reagent with 10 µg of glycogen as a carrier. The OrR RNA samples were resuspended in 53 µl of DEPC-treated H₂O and

supplemented with 6 μ l of a 10X DNase buffer (400 mM Tris-HCl [pH 7.5], 60 mM MgCl_2). The RNA from mutant or wild-type sibling pools was resuspended in 26 μ l of DEPC-treated H_2O , then supplemented with 3 μ l of the same 10X DNase buffer, and then 1 μ l (10 u) of RNase-free DNase (Amersham Biosciences) was added to each of the RNA samples. The reaction mixtures were incubated at 37°C for 15 minutes, followed by a 5-minute incubation at 60°C to inactivate the DNase. The volume of each reaction mixture was then increased to 200 μ l with DEPC-treated H_2O and extracted once with 200 μ l of phenol:chloroform:isoamyl alcohol (25:24:1) and once with 200 μ l of chloroform:isoamyl alcohol (24:1). The aqueous phase was placed in a new microcentrifuge tube, mixed with 0.1 volumes of 3.0 M sodium acetate (pH 5.2) and 2.5 volumes of ice cold 95% ethanol, and placed at -20°C for 30 minutes. Next, the RNA samples were pelleted by centrifugation at 12,000 X g for 10 minutes at 4°C. They were then washed with ice cold 70% ethanol, air dried, and resuspended in 20 μ l of DEPC-treated H_2O . The concentration of each RNA sample was determined by diluting 4 μ l of each sample into 1 ml of DEPC-treated water and measuring the optical density of the resulting solution at 260 nm. The remainder of each RNA sample was stored at -70°C for later use.

Reverse transcription

Primers were designed to facilitate the reverse transcription of the *Ddc* (5'-AAT-CGCTCCACTCAGCATGT-3') and *rpL32* (5'-TCTTCTTGAGACGCAGGCGA-3') mRNAs. The RNA samples were placed in 0.5 ml microcentrifuge tubes. To this, 5

pmol of each primer and DEPC-treated H₂O were added to a final volume of 12 μ l. The mixtures were then incubated for 10 minutes at 70°C, quick chilled on ice, and briefly centrifuged to collect the contents of the tube. Next, 4 μ l of 5X First Strand Buffer, 2 μ l of 0.1 M DTT, 1 μ l of 10 mM dNTP mix, and 1 μ l (200 u) of Superscript II (Invitrogen) reverse transcriptase were added to the RNA samples. The reaction mixtures were incubated for 60 minutes at 45°C, followed by a 10-minute incubation at 95°C to inactivate the reverse transcriptase. Finally, the RNA samples were quick chilled on ice, briefly centrifuged, and then frozen at -20°C.

PCR

A 10X solution of PCR buffer was prepared ahead of time containing: 500 mM Tris-HCl (pH 8.2), 15 mM MgCl₂, 500 mM KCl, 0.01% gelatin, and 2 mM dNTPs (each). Primers were designed to amplify the first strand cDNA products of the *Ddc* (forward, 5'-GCCAAGCGCACAGCAATCAG-3'; reverse, 5'-ATGACTCGCTCG-ATGTCCTG-3') and *rpL32* (forward, 5'-AGCATAACAGGCCCAAGATCG-3'; reverse, 5'-AGTAAACGCGGGTTCTGCAT-3') mRNAs. Each of the PCR reaction mixtures was composed of 3 μ l of the reverse transcription mix, 1X PCR Buffer, 10 pmol of *Ddc*-specific primers (each), 5 u Taq DNA polymerase and H₂O to a final volume of 28 μ l. These reaction mixtures were run through the first three cycles of the PCR program. The program was then paused, 10 pmol of the *rpL32*-specific primers (each) were added to the reaction mixtures, and the program was run to completion. The final PCR reaction conditions were as follows: one cycle of 95°C

for 3 minutes, 62°C for 1 minute, and 74°C for 2 minutes; 23 cycles of 94°C for 1 minute, 62°C for 1 minute, and 74°C for 2 minutes. When testing the linearity of the PCR, the reaction conditions were extended from 23 to 27 cycles in order to reach the plateau phase of the PCR reaction.

Analysis of PCR products

A 3 µl aliquot of 10X Orange G loading buffer (0.3% Orange G, 25% Ficoll [Type 400], 100mM EDTA) was added to the completed PCR reaction mixtures, 28 µl of each reaction mixture was loaded into a 2% TAE agarose gel and electrophoresed at 110 volts for 2 to 2.5 hours in 1X TAE buffer. The gel was then placed in 500 ml of a 0.5 µg/ml ethidium bromide solution and allowed to stain for 1 hour under constant agitation. The gel was then placed in 500 ml of distilled H₂O and allowed to destain for 1 hour under constant agitation. The stained gel was photographed using an Appligene video imager (Oncor) and stored as a TIFF file for computer analysis. The TIFF file was downloaded into the Gel-Pro Analyzer program (Media Cybernetics), which graphed the bands as peaks of ethidium bromide staining and provided the integrals of the peak areas.

Analysis of LacZ expression in larval epidermis and imaginal discs

Isolation of the epidermis and imaginal discs from early third instar larvae was done in a solution of phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, [pH 7.4]). Briefly, larvae were grasped at roughly the

midpoint of the anterior-posterior axis with two pairs of needle nose tweezers and separated into anterior and posterior halves. The anterior portion of each larva was then turned inside-out while ensuring that the imaginal discs and mouthparts remained attached to the epidermis and placed in a fixing solution (0.75% glutaraldehyde in PBS) for 20 minutes. The glutaraldehyde solution was removed from the larval tissues by washing three times in PBT (0.1% Triton X-100 in PBS). Next, the larval tissues were placed in a freshly prepared LacZ staining buffer (10 mM sodium phosphate [pH 7.2], 150 mM NaCl, 1mM MgCl₂, 5mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.1% X-gal in N,N-dimethylformamide) and placed at 37°C for a period of 1 hour. Washing three times in PBT terminated the reaction and the tissue was then stored at 4°C in PBS for a period of no longer than seven days. The epidermis and imaginal discs of the larvae were mounted on microscope slides in 80% glycerol and photographed using a Zeiss Axiophot microscope.

Analysis of LacZ expression in larval extracts

Drosophila larvae were collected individually, inspected to determine their genotype, and weighed to determine whole body mass. Each organism was then flash frozen in liquid nitrogen and stored at -70°C. When numbers permitted, three to four organisms of each genotype were pooled and each pool was placed into a single microcentrifuge tube. Homogenization buffer (50 mM potassium phosphate [pH 7.5], 1 mM MgCl₂, 0.01 mM DTT) was freshly prepared, 150 µl aliquots were added to each pool of larvae, and the organisms were homogenized using a plastic micropestle.

The protein extracts were cleared by centrifugation, placed on ice, and 25 μ l aliquots, or an appropriate dilution in homogenization buffer containing 0.1 mg/ml bovine serum albumin, were combined with 180 μ l of assay buffer (0.4% ONPG in homogenization buffer) that had been pre-warmed to 37°C. A simultaneous control reaction was prepared that contained 25 μ l of larval extract and 180 μ l of homogenization buffer. All of the reaction mixtures were placed in a 37°C water bath for 60 minutes, followed by the addition of 600 μ l of 1M Na₂CO₃ to stop the reaction. The amount of β -galactosidase activity in each reaction was determined by measuring the optical density (O.D.) of each reaction product at a wavelength of 420 nm, with the control reaction serving as a blank. Finally, the β -galactosidase activity in each assay was standardized by dividing the O.D. by the measurement of total protein in the respective assay.

Plasmid DNA constructs

The plasmid constructs used in this study are shown in Table II-1.

Plasmid DNA preparation

The majority of plasmid DNA mini preparations were carried out by the alkaline lysis method in Ausubel *et al.* (1994) with the following deviations: following neutralization, the mixture containing plasmid DNA was centrifuged for 5 minutes to remove genomic DNA and cell debris; the supernatant from that spin was then

Table II-1: Plasmid Constructs

Plasmid name	Source	Reference
P[<i>Ddc-lacZ</i>]SH	Li Chen	Chen <i>et al.</i> (2002)
P[<i>Ddc-lacZ</i>]BH	Li Chen	Chen <i>et al.</i> (2002)
P[<i>Ddc-lacZ</i>]RH	Li Chen	Chen <i>et al.</i> (2002)
pPal1-SX-188-cc-luc	Peter Cherbas	Unpublished. Ecdysone-inducible luciferase reporter vector
pCMX-EcRA	Peter Cherbas	Unpublished. EcR-A expression vector
pCMX-EcRB1	Peter Cherbas	Unpublished. EcR-B1 expression vector
pCMX-EcRB2	Peter Cherbas	Unpublished. EcR-B2 expression vector
pPac-PL	Carl Thummel	Krasnow <i>et al.</i> (1989). Expression vector for <i>Drosophila melanogaster</i> cell lines.
pBluescribedm527	Cynthia Bayer	DiBello <i>et al.</i> (1991). dm527 cDNA clone contained in the Bluescribe vector.
pBS(-)SKCD5	Cynthia Bayer	DiBello <i>et al.</i> (1991). CD5 cDNA clone contained in the Bluescript II KS (-) vector.
pBSIIKS(+)dm797	Cynthia Bayer	DiBello <i>et al.</i> (1991). dm797 cDNA clone contained in the Bluescript SK (-) vector.
pBS(-)SK28-I	Cynthia Bayer	Bayer <i>et al.</i> (1996). 28-I cDNA clone contained in the Bluescript SK (-) vector.
pPac-PL(Z1)	This study	
pPac-PL(Z2)	This study	
pPac-PL(Z3)	This study	
pPac-PL(Z4)	This study	

extracted once with phenol:chloroform:isoamyl alcohol (25:24:1); and following ethanol precipitation the plasmid DNA was resuspended in TE (pH 8.0) containing 20 µg/ml RNase A.

Mini preparations of plasmid DNA to be used for ligation experiments were done using the QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's protocol.

Restriction Enzyme Digestion

All restriction enzyme digestions were carried out in 20 µl reaction volumes using Invitrogen enzymes according to the manufacturer's instructions. All digestions were terminated by heat inactivating the reactions according to the manufacturer's instructions.

Modification of DNA molecules

When necessary, the 5' phosphate groups of linearized vector molecules were removed by treatment with Thermosensitive Alkaline Phosphatase (Invitrogen) according to the manufacturer's simplified protocol. When necessary, DNA molecules were made to be blunt ended by adding 1 µl (5 u) T4 DNA polymerase (Invitrogen) and 1 µl of 2 mM dNTPs (each) to the heat inactivated restriction enzyme digestion and incubating for 5 minutes at 37°C. This was followed by a 10-minute incubation at 75°C to heat inactivate the T4 DNA polymerase.

Purification of DNA molecules

Following restriction enzyme digestion and any necessary modification reactions, the DNA molecules were separated by electrophoresis in a 0.8% TAE agarose gel for between 1 and 2 hours. Staining of the gel was accomplished by running the gel in 1X TAE buffer containing 0.5 µg/ml of ethidium bromide. The DNA was visualized using a UV transilluminator and the desired bands were excised from the gel. The excised bands were purified using the GeneClean II kit (Bio 101) according to the manufacturer's protocol.

Ligation of DNA molecules

Recombinant molecules containing the desired DNA fragments were generated using T4 DNA ligase (Promega) according to the manufacturer's protocol. All ligation reactions were incubated for 24 hours at 15°C, followed by a 10-minute incubation at 70°C to heat inactivate the T4 DNA ligase. The reaction mixtures were allowed to cool to room temperature and they were then transformed into competent *E. coli* DH5α cells according to the protocol of Ausubel *et al.* (1994). The cells were plated onto LB-agar plates containing 100 µg/ml of ampicillin (Sigma-Aldrich) and incubated overnight at 37 °C. A subset of the colonies found growing on each plate was selected and the plasmid DNA isolated from each one using the alkaline lysis plasmid DNA mini preparation protocol (see above). The plasmids were then digested with restriction enzymes (see protocol above) that would allow for the detection of the appropriate insert and a determination of its size and orientation. One

of the colonies deemed to have the correct plasmid was selected and the remainder of the 5 ml culture from that mini preparation was used to make a glycerol stock and streak out a new plate.

Preparation of plasmid DNA for cell culture transfections

Large-scale preparations of plasmid molecules were done using either the Qiagen Midi Prep kit or the Qiagen Maxi Prep kit. All large-scale preparations were started from a single, isolated colony and performed according to the manufacturer's protocol.

The identity of the BR-C expression constructs was confirmed by PCR amplification at this point. Briefly, in the PCR reactions Qiagen Taq polymerase was used according to the protocol designed for use with Q-solution. The reactions utilized a core primer (5'-ACAAGATGTTCCATGCAGCC-3'), the appropriate Z1 (5'-TGC-TGGTGCTGCTGGTGATA-3'), Z2 (5'-TCATCTCCATTTGCGCGGGA-3'), Z3 (5'-GATGGCGGTCGTCTTAAGCA-3'), and Z4 (5'-GTGGTTGTTTCAGCGAGT-TCA-3') primers, and cycling parameters as described previously (Hodgetts *et al.*, 1995).

Maintenance of cells

The *Drosophila melanogaster* L57-3-11 (Cherbas *et al.*, 1992) cells were maintained in 15 ml of HyQ-CCM3 medium (Hyclone) contained in a tissue culture-grade 75

cm² T-flask. Flasks of cells were kept inside Rubbermaid containers at 25 °C with air as a gas phase. The cells were transferred to new T-flasks every 4 days, keeping them within their exponential growth phase ($\sim 1 \times 10^6$ to 1×10^7 cells/ml).

Setup for transfection

The transfection process was initiated by collecting cells at the dense end of the exponential growth phase (typically between 0.8×10^7 cells/ml to 1.2×10^7 cells/ml). 1 ml of the culture was used to obtain an accurate cell count with a haemocytometer. The remaining 13 ml of the culture was placed in a conical tube, the cells pelleted by centrifugation at 1000 rpm for 10 minutes, and resuspended in an equal volume of fresh HyQ-CCM3 medium. Aliquots of the culture were then placed into a total of 15 ml HyQ-CCM3 medium such that the cells were at a concentration of 1×10^6 cells/ml. A single 75 cm² T-flask was prepared for each combination of constructs to be transfected. The cells were then incubated at 25°C for a period of 48 hours.

Electroporation

After the 48-hour incubation, the cells were collected from the T-Flasks, pelleted, washed twice in one-half the original volume of medium, and resuspended in 1 ml of HyQ-CCM3 medium. A single BTX 0.4 cm gap cuvette (Genetronics, Inc.) was set up for each combination of plasmids to be transfected. Initially, 10 µg of the appropriate EcR expression vector and 2 µg of the appropriate reporter plasmid were added to the cuvette. When performing triple transfections, 9 µg of the appropriate

EcR expression vector, 9 μg of the appropriate BR-C expression vector, and 2 μg of the reporter plasmid were added to the cuvette. Mock transfections were performed by substituting the plasmid solutions with an equal volume of the plasmid solvent TE. In all cases, 800 μl of the resuspended cells from a single plate was added to each electroporation cuvette and the mixture let set for 5 minutes. Next, the cells were shocked with a BTX model ECM 630 electroporator (Genetronics, inc.) at 440 V/cm, 1200 μF , and 0 Ω , then allowed to recover for 10 minutes. Each 800 μl suspension of cells was then transferred into 9.2 ml of HyQ-CCM3 medium contained in a tissue culture-grade 25 cm^2 T-Flask.

Protein expression and ecdysone induction

A stock solution of 20-hydroxyecdysone (Sigma-Aldrich) was prepared by dissolving the lyophilized powder in filter sterilized 95% ethanol at a concentration of 10 mg/ml and then stored at -70°C . Working solutions of 20-hydroxyecdysone (1 mg/ml) were prepared by mixing 1 part of the stock solution with 9 parts of HPLC-grade H_2O . The solvent control was prepared by diluting 1 part of filter sterilized 95% ethanol into 9 parts of HPLC-grade H_2O . The transfected cells were left at 25°C for a period of 24 hours to allow for sufficient levels of protein expression and each culture was then split into two equal aliquots of 5 ml each. One aliquot of the cells from each culture was treated with 5 μl of the 1 mg/ml solution of 20-hydroxyecdysone and the other aliquot treated with 5 μl of the ethanol solvent

solution. The cells were then incubated at 25°C for a period of 24 hours to allow for 20-hydroxyecdysone-induced expression from the reporter plasmids.

Analysis of L57-3-11 cells transfected with the luciferase expression construct

After the 24-hour induction period, the cells were collected and the protein fraction was extracted using Cell Culture Lysis Reagent (Promega) according to the manufacturer's protocol. The level of luciferase activity was then measured using the Luciferase Assay System (Promega) according to the manufacturer's protocol with an LKB luminometer.

Analysis of L57-3-11 cells with LacZ expression constructs

After the 24-hour induction period, the cells were collected and the protein fraction was extracted according to Koelle *et al.* (1991) with the following exceptions: a total of 4 ml of cells was harvested from each 25 cm² T-Flask and the cells were resuspended in a total of 200 µl of freeze/thaw buffer. The protein concentration of each extract was determined using Bio-Rad's Protein Assay reagent according to the protocol supplied by the manufacturer with bovine serum albumin serving as the standard. The remainder of the protein extract was frozen at -70°C for a period of no longer than seven days. β-galactosidase activity was measured by combining 20 µl of protein extract, or an appropriate dilution in freeze/thaw buffer containing 0.1 mg/ml bovine serum albumin, with 180 µl of assay buffer (see above) and incubating the

mixture at 37°C for a period of 30 minutes. A control reaction, containing 20 µl of protein extract and 180 µl of homogenization buffer, was also set up and incubated alongside the experimental reactions. At the end of the 30-minute incubation, the reactions were terminated by adding 600 µl of 1M Na₂CO₃. The O.D. at 420 nm of the reaction products was then measured with a spectrophotometer (LKB), with the control reaction serving as a blank. Lastly, the β-galactosidase activity in each assay was standardized by dividing the O.D. by the µg quantity of total protein used in the respective assay.

Results

The role of the EcR-B1 protein in the process of *Ddc* expression at pupariation

Bender *et al.* (1997) have isolated two mutations, termed *EcR*^{Q50st} and *EcR*^{W53st}, which are specific to the EcR-B1 protein isoform. Both mutations are caused by premature stop codons generated within the EcR-B1-specific exon 2, resulting in truncated EcR-B1 proteins that lack both the DNA-binding domain (DBD) and the ligand-binding domain (LBD). Bender *et al.* (1997) have placed the *EcR*^{Q50st} and *EcR*^{W53st} alleles in a nonpupariating class of *EcR* mutations to describe their lethality at the onset of metamorphosis. More specifically, these mutant organisms reach the third instar stage of development and initiate wandering but subsequently fail to shorten, attach themselves to a solid surface, or, most importantly, harden their cuticle. Furthermore, previous research has shown that the epidermis, the site of the majority of DDC production at pupariation, predominantly expresses the EcR-B1 protein isoform (Talbot *et al.*, 1993). Taken together, these lines of evidence suggest that the EcR-B1 protein mediates ecdysone-induced expression of the *Ddc* gene at pupariation. To test this hypothesis, a coupled reverse transcription-polymerase chain reaction (RT-PCR) assay was used to measure the steady state *Ddc* mRNA levels in *EcR-B1* mutant organisms and wild-type sibling organisms.

The validity of the RT-PCR assay depends upon certain pre-established parameters.

The accuracy of any RT-PCR assay depends upon two critical parameters. First, the assay must generate a product that is directly proportional to the quantity of input RNA. Second, the PCR portion of the assay must be in the linear range of amplification when it is terminated for product quantification. To demonstrate that the RT-PCR assay met these requirements, RNA was isolated from three sets of Oregon R (OrR) white prepupae, termed sample 11, sample 12, and sample 13, for a series of control experiments. The white prepupae are larvae that are in the early stages of puparium formation and provide an RNA sample with the maximal *Ddc* mRNA expression levels necessary for these control experiments.

The proportionality of the RT-PCR assay to the quantity of input RNA was determined by performing a set of experiments with each experiment consisting of a series of four reaction mixtures containing 75, 125, 175, and 225 ng respectively of total RNA. These values were selected because they encompassed the 100ng chosen as the amount of input RNA to be used when measuring expression levels in the *EcR-B1* mutant organisms. In the initial experiment, each of the four reaction mixtures was prepared with RNA from sample 11. The reaction mixtures were reverse transcribed and then amplified for a total of 23 cycles. Next, the *Ddc* and *rpL32* reaction products were quantified and plotted on a graph as a function of the amount of input RNA (Fig. III-1). This process was duplicated with RNA from samples 12 and 13 and the quantities of these *Ddc* and *rpL32* reaction products were also plotted as a function of the amount of input RNA (Figs. III-2, III-3). The curves for all three

Figure III-1 Determining the linearity of input RNA from sample 11. A series of four reaction mixtures was prepared containing 75, 125, 175, and 225 ng respectively of sample 11 RNA. The reaction mixtures were then reverse transcribed, amplified using the PCR, and quantified in arbitrary units as described in Materials and Methods. These values, which represent the integral of the band density, were then plotted on a graph as a function of input RNA.

Linearity of input RNA for sample 11

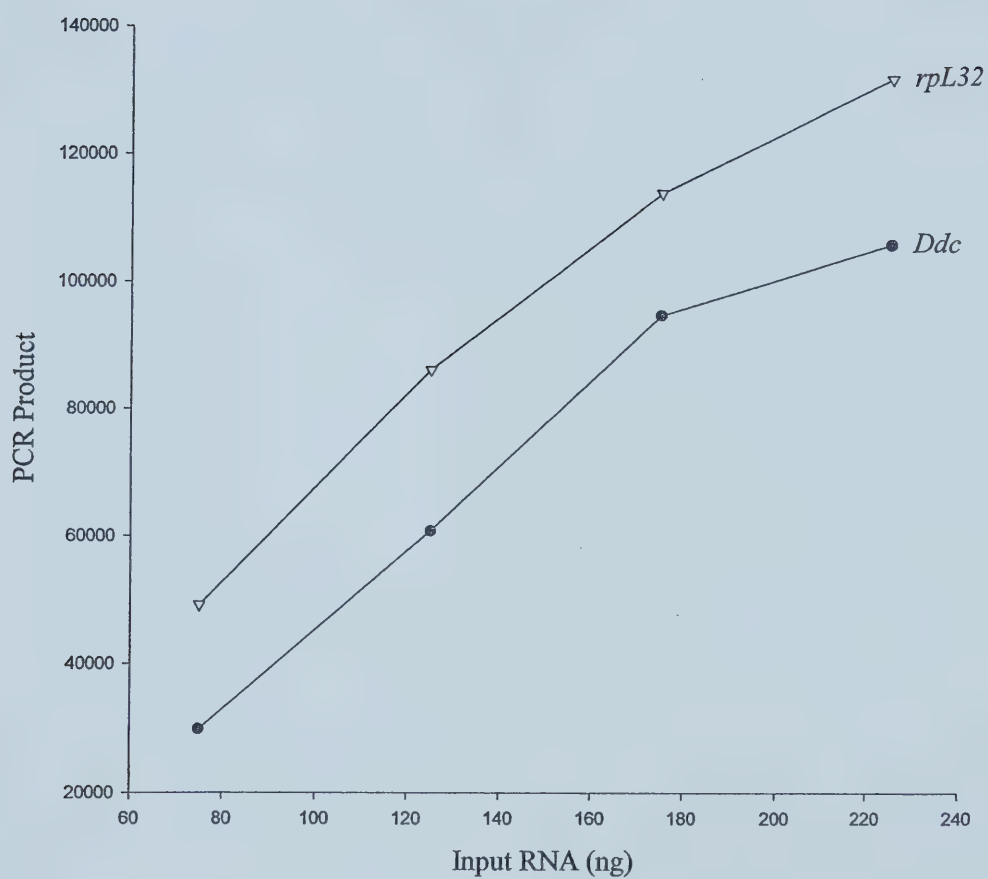


Figure III-2 Determining the linearity of input RNA from sample 12. A series of four reaction mixtures was prepared containing 75, 125, 175, and 225 ng respectively of sample 12 RNA. The reaction mixtures were then reverse transcribed, amplified using the PCR, and quantified in arbitrary units as described in Materials and Methods. These values, which represent the integral of the band density, were then plotted on a graph as a function of input RNA.

Linearity of input RNA for sample 12

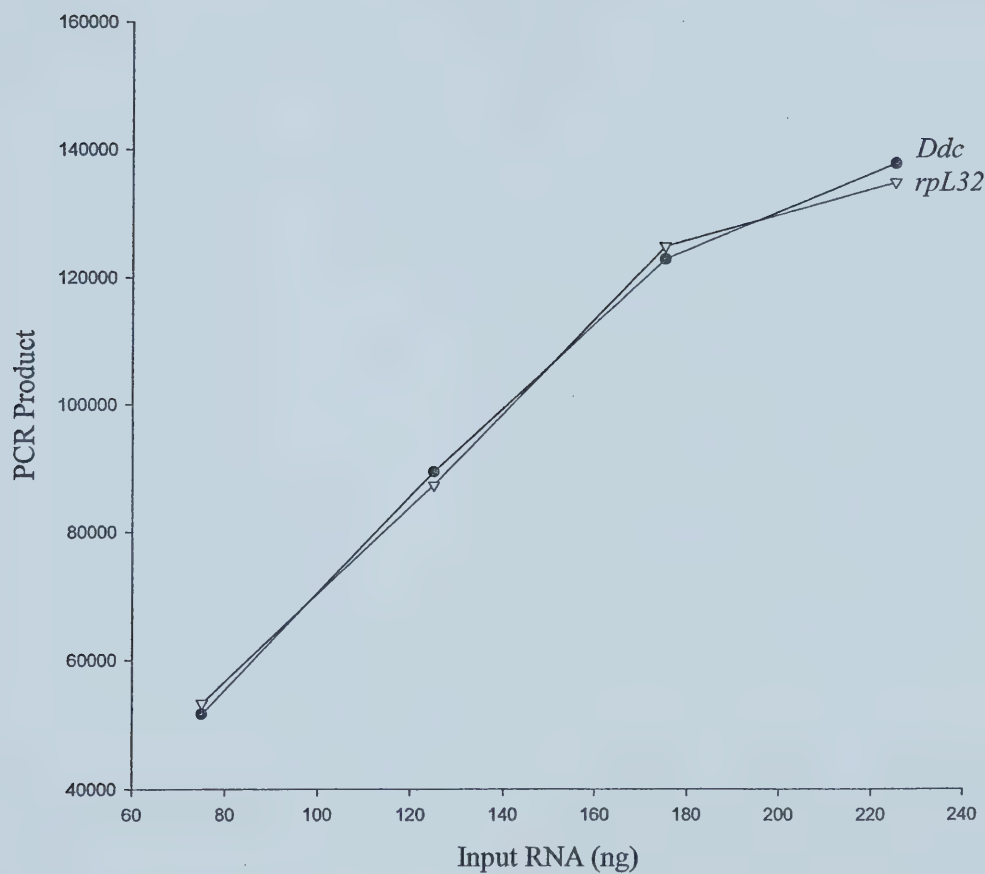
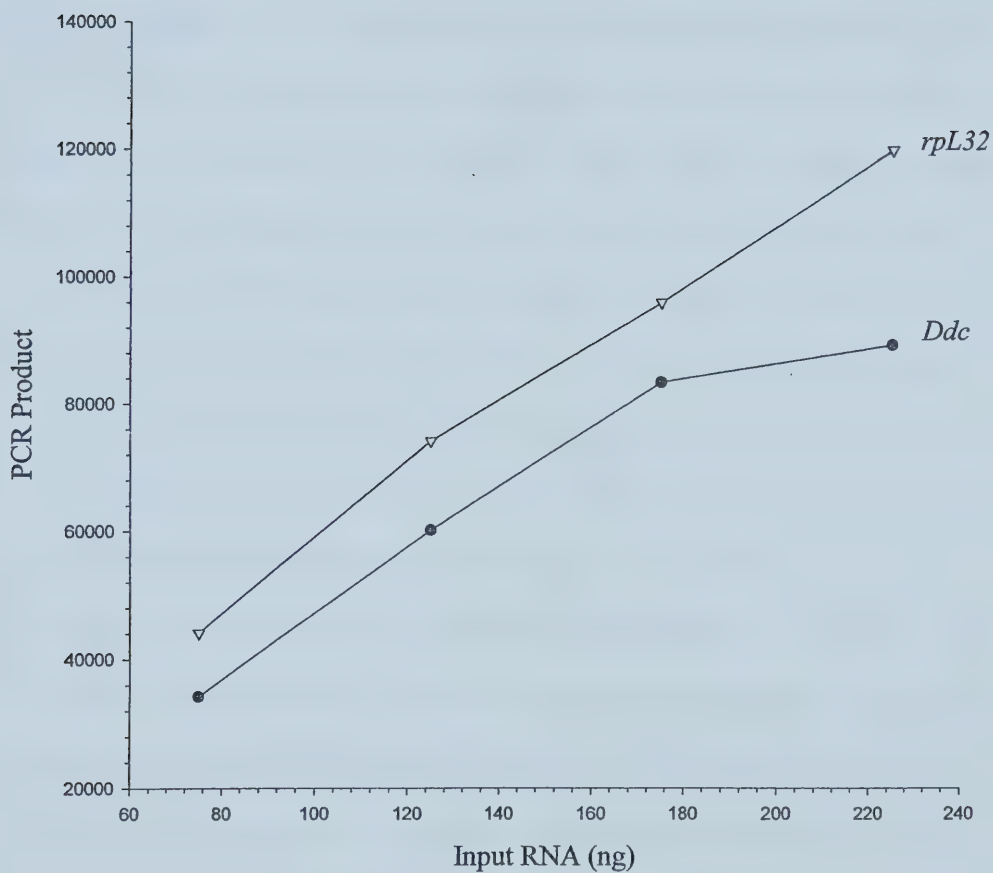


Figure III-3 Determining the linearity of input RNA from sample 13. A series of four reaction mixtures was prepared containing 75, 125, 175, and 225 ng respectively of sample 13 RNA. The reaction mixtures were then reverse transcribed, amplified using the PCR, and quantified in arbitrary units as described in Materials and Methods. These values, which represent the integral of the band density, were then plotted on a graph as a function of input RNA.

Linearity of input RNA for sample 13

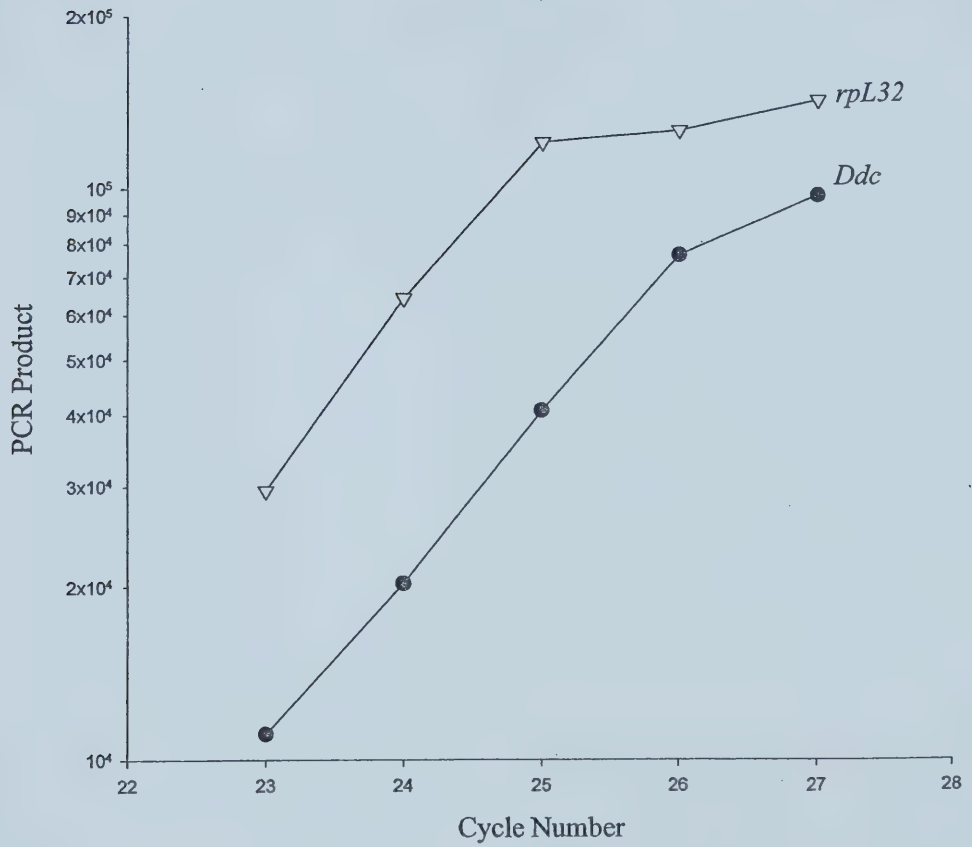


graphs were linear between 75 and 175 ng of input RNA, thus demonstrating that the RT-PCR assay was proportional to the amount of input RNA around the range of 100 ng of input RNA used in the final experimental procedure.

The linear phase of the PCR portion of the assay was determined by performing a set of experiments with each experiment consisting of a series of five identical reaction mixtures, each containing 100 ng of input RNA. In the initial experiment, RNA from sample 11 was used to prepare the five reaction mixtures, which were then reverse transcribed and amplified under standard conditions for a total of 23 cycles. At the end of the 23rd cycle, one of the reaction mixtures was removed from the thermal cycler and the reaction was terminated by adding Orange G loading buffer. At the end of the 24th cycle, a second reaction mixture was removed from the thermal cycler and the reaction was terminated by adding Orange G loading buffer. This process was repeated until the final reaction mixture was removed after the completion of the 27th cycle. The *Ddc* and *rpL32* reaction products were then quantified and plotted on a semi-logarithmic graph as a function of the number of PCR amplification cycles (Fig. III-4). This experiment was repeated using RNA from samples 12 and 13, the reaction products from these experiments were also quantified and then plotted on semi-logarithmic graphs as a function of the number of PCR amplification cycles (Figs. III-5, III-6). The curves for all three graphs were linear between 23 and 25 amplification cycles, thus demonstrating that the RT-PCR assay would be in the linear range of amplification when it was terminated for quantification after 23 cycles of amplification.

Figure III-4 Determining the linearity of PCR reaction from sample 11. A series of five identical reaction mixtures was prepared, each containing 100 ng of sample 11 RNA. The reaction mixtures were then reverse transcribed, amplified using the PCR, and quantified in arbitrary units as described in Materials and Methods and Results. These values, which represent the integral of the band density, were then plotted on a semi-logarithmic graph as a function of the number of PCR amplification cycles.

Linearity of PCR for RNA sample 11



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Figure III-5 Determining the linearity of PCR reaction from sample 12. A series of five identical reaction mixtures was prepared, each containing 100 ng of sample 12 RNA. The reaction mixtures were then reverse transcribed, amplified using the PCR, and quantified in arbitrary units as described in Materials and Methods and Results. These values, which represent the integral of the band density, were then plotted on a semi-logarithmic graph as a function of the number of PCR amplification cycles.

Linearity of PCR for RNA sample 12

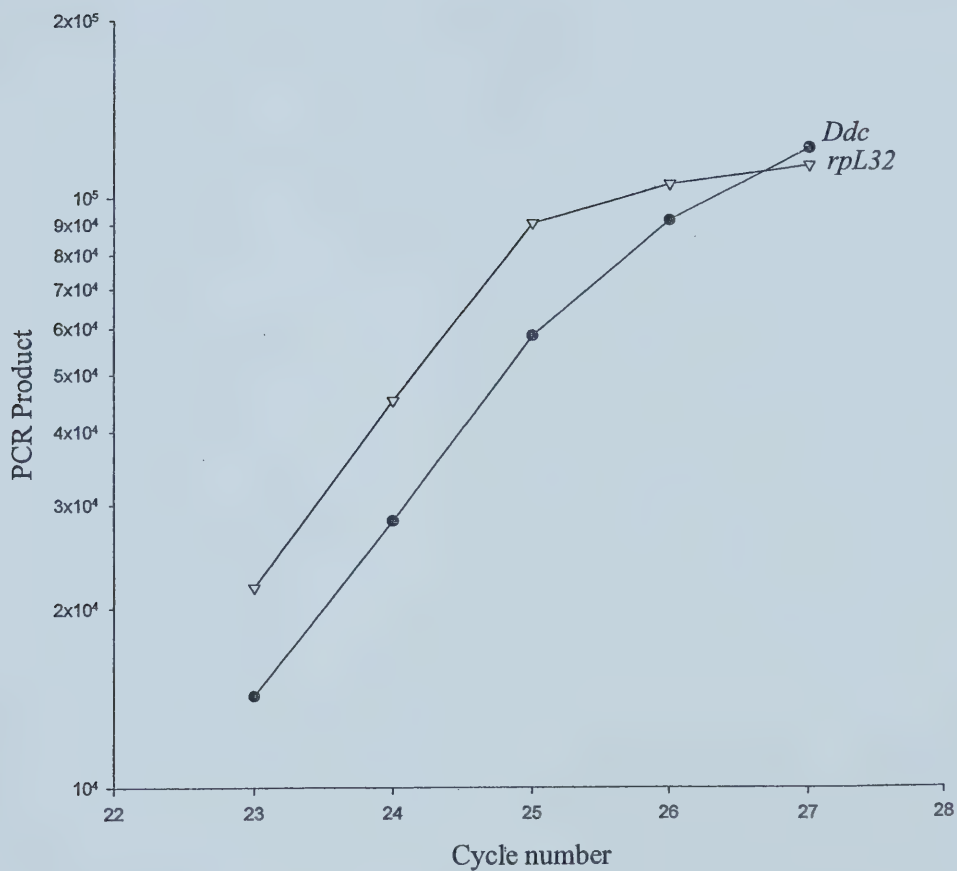
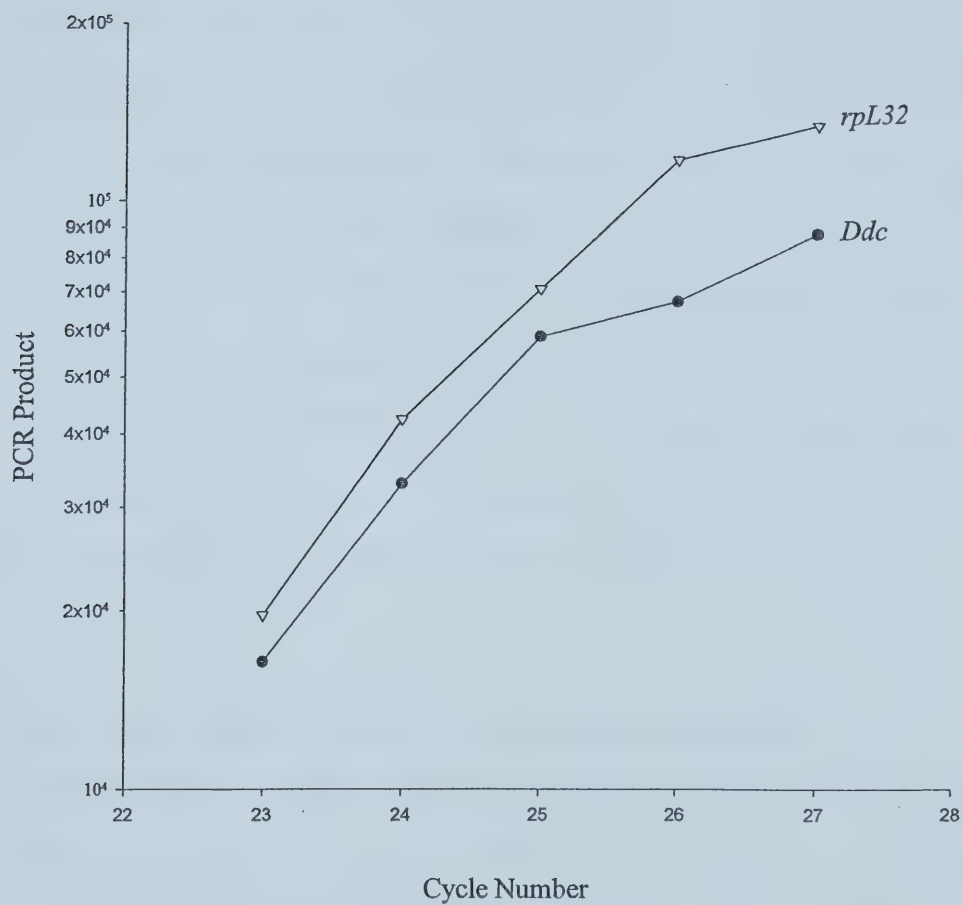


Figure III-6 Determining the linearity of PCR reaction from sample 13. A series of five identical reaction mixtures was prepared, each containing 100 ng of sample 13 RNA. The reaction mixtures were then reverse transcribed, amplified using the PCR, and quantified in arbitrary units as described in Materials and Methods and Results. These values, which represent the integral of the band density, were then plotted on a semi-logarithmic graph as a function of the number of PCR amplification cycles.

Linearity of PCR for RNA sample 13



The stocks carrying the EcR^{Q50st} and EcR^{W53st} alleles are crossed to the EcR^{M554fs} null mutation to generate mutant organisms.

Because the EcR^{Q50st} and EcR^{W53st} alleles are both recessive lethal mutations, they were maintained as a balanced stock opposite a CyO , y^+ chromosome. This required that a cross be made to generate $EcR-B1$ mutant organisms, as detailed in Figure III–7. Briefly, organisms from the stocks bearing the EcR^{Q50st} and EcR^{W53st} alleles were mated individually to organisms from a stock bearing the EcR^{M554fs} null allele, as per Bender *et al.* (1997). These crosses generated the $EcR-B1$ mutant larvae (yw ; $EcR^{Q50st} / EcR^{M554fs}$ or yw ; $EcR^{W53st} / EcR^{M554fs}$), which were identified on the basis of their *yellow* phenotype and gathered for analysis. At the same time, *yellow*⁺ larvae were gathered to use as control samples. The *yellow*⁺ organisms all carried a fully functional copy of the *EcR* gene and, because they were collected alongside the *yellow* samples, served as identically staged wild-type larvae.

The $EcR-B1$ mutations abolish *Ddc* expression at pupariation.

Two independent crosses were made with both the EcR^{Q50st} and EcR^{W53st} alleles. The resulting progeny were collected one hour after gut clearing (Andres and Thummel, 1994) and following isolation of total RNA, the level of *Ddc* expression in each sample pool was measured by RT-PCR analysis. The final reaction parameters for the RT-PCR assay consisted of 100 ng of input RNA and 23 amplification cycles. The results of these assays, shown in Figure III–8, indicate that the wild-type organisms were expressing high levels of *Ddc* mRNA. This is consistent with the

Figure III–7 Generation of *EcR-B1* mutant organisms. The *EcR-B1* mutant organisms were generated by crossing either *yw; EcR^{W53st}/CyO, y⁺* (shown) or *yw; EcR^{Q50st}/CyO, y⁺* virgin females to males with a *yw; EcR^{M554fs}/CyO, y⁺* genotype. The *EcR-B1* mutant offspring, which lack the *CyO, y⁺* chromosome, were selected on the basis of their *yellow* phenotype, which could be scored in the mouthparts. The wild-type siblings, which were *yellow⁺* because they carry the *CyO, y⁺* chromosome, were also collected to serve as a control.

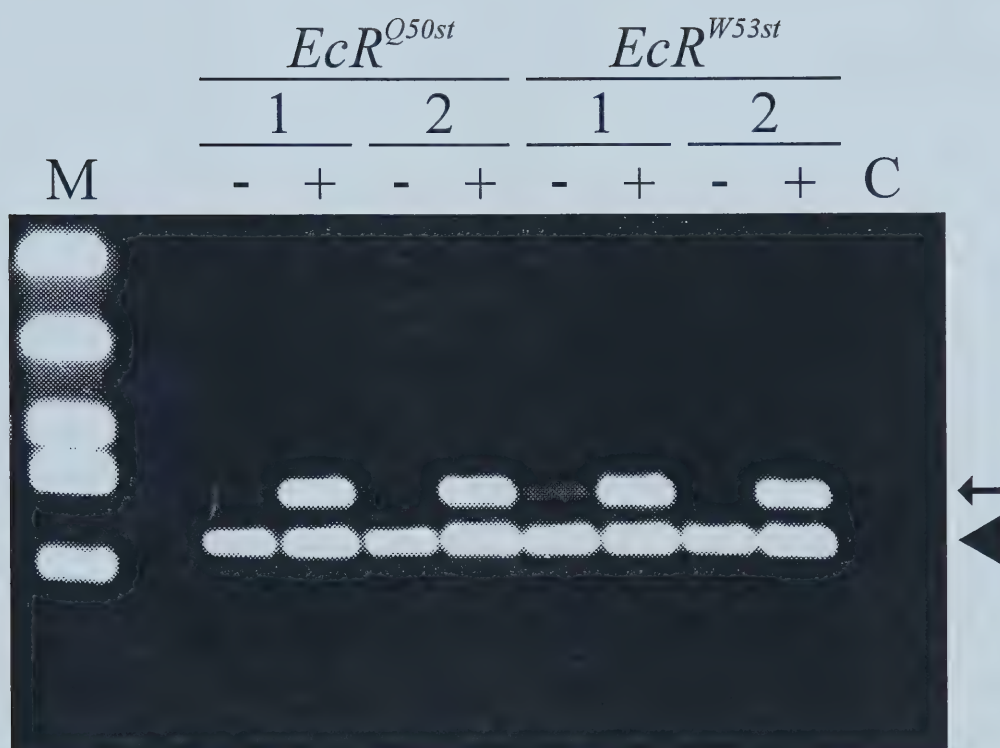
$$\text{♀ } yw; \frac{EcR^{W53st}}{CyO, y^+} \times \text{♂ } yw; \frac{EcR^{M554fs}}{CyO, y^+}$$



$$yw; \frac{EcR^{W53st}}{EcR^{M554fs}} \left] \text{ yellow phenotype} \right.$$

$$\begin{array}{l} yw; \frac{EcR^{W53st}}{CyO, y^+} \\ yw; \frac{EcR^{M554fs}}{CyO, y^+} \end{array} \left] \text{ yellow}^+ \text{ phenotype} \right.$$

Figure III-8 Comparative RT-PCR analysis of *Ddc* expression in *EcR-B1* and wild-type organisms at pupariation. Two independent sets of organisms were collected for each *EcR-B1* allele (indicated as 1 and 2). Total RNA was isolated from sample organisms and then reverse transcribed and amplified using *Ddc*-specific and *rpL32*-specific primers. The expression levels of *Ddc* mRNA (arrow) and *rpL32* mRNA (arrowhead) in mutant (–) and wild-type (+) progeny organisms from the indicated *EcR-B1* parental alleles are shown. M, marker DNA.



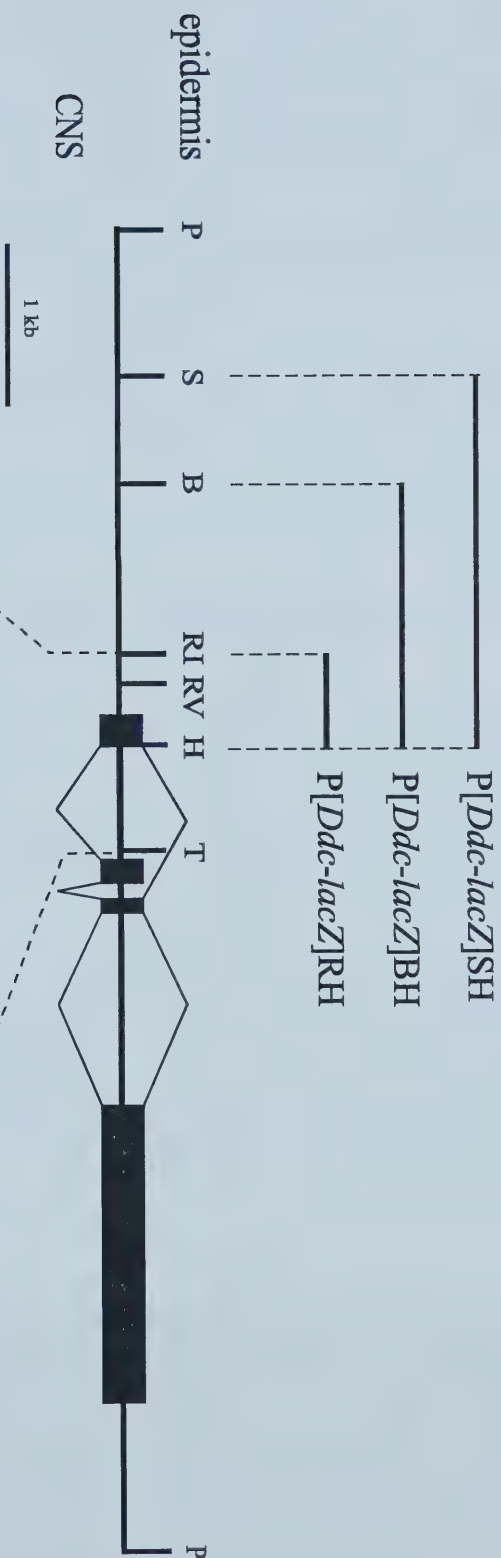
fact that these wild-type organisms have been shown to sclerotize their cuticle and undergo the normal metamorphic process. On the other hand, the *Ddc* reaction products generated by the RT-PCR assays of both the two *EcR*^{Q50st} and two *EcR*^{W53st} mutant samples are not even detected by the image analysis software. This almost complete lack of *Ddc* mRNA expression in the *EcR*^{Q50st} and *EcR*^{W53st} mutant samples clearly demonstrates that it is the EcR-B1 isoform that mediates the induction of the gene at pupariation.

An analysis of the *Ddc* regulatory region in an *EcR-B1* mutant background

Recent work has uncovered an ecdysone response element (EcRE) and a silencing element(s) in the 5' region of the *Ddc* gene (Chen *et al.*, 2002a). Additional evidence indicates that the *BR-C* gene exerts its effect through a *cis*-acting element in the 5' region of the *Ddc* gene (Chen *et al.*, 2002b). The bulk of these results is based on work where fragments of the 5' region of the *Ddc* gene, shown in Figure III-9, were fused to the P{CaSpeR-AUG-β-gal} LacZ reporter gene (Thummel *et al.*, 1988), followed by introduction of the constructs into *Drosophila* via P-element mediated transformation. Chen *et al.* (2002a) have shown that the deletion of a small segment of DNA between -97 and -83 bp relative to the *Ddc* transcription start site drastically reduces ecdysone-driven transcription of the *Ddc* gene. This led the authors to conclude that the ecdysone response element for the *Ddc* gene, termed EcRE₋₉₇, lies in this region, which places it inside the -382 bp to +174 bp boundary of the *EcoR* I to *Hpa* I construct. Also, Chen *et al.* (2002a) demonstrated that the constructs lacking

Figure III–9 Diagram of the *Ddc-lacZ* constructs used in this thesis. (A) The 7.6 kb genomic fragment of the *Ddc* gene that has been shown to be sufficient for normal temporal and spatial expression patterns (Scholnick *et al.*, 1983). The neural- and epidermal-specific splicing patterns of the transcription unit are shown, as are the restriction sites for *Pst* I (P), *Sca* I (S), *Bsm* I (B), *EcoR* I (RI), *EcoR* V (RV), *Hpa* I (H), and *Taq* I (T). The various fragments of the *Ddc* gene used to drive reporter gene expression are shown above the 7.6 kb genomic fragment, along with the associated name of each reporter construct. (B) An enlarged diagram of the *EcoR* I–*Taq* I fragment of the *Ddc* gene that shows the location of the EcRE_{–97} (Chen *et al.*, 2002a).

(A)



the *Sca* I to *Bsm* I fragment of the *Ddc* gene generated precocious β -galactosidase activity in third instar larvae, indicating that a silencer element or elements must lie in this region. Lastly, additional evidence indicates that the *Bsm* I to *Eco*R I fragment of the *Ddc* gene contains a *cis*-acting element(s) that confers responsiveness to *BR-C* inputs (Chen *et al.*, 2002b). Fortunately, stocks carrying these constructs are readily available and this allowed us to ask questions regarding the role of the EcR-B1 protein in the context of these previously mentioned findings.

Stocks carrying an *EcR-B1* mutation and the relevant LacZ reporter constructs must be created prior to experimental analysis.

To study these LacZ reporter constructs in an *EcR-B1* mutant background, it was necessary to create stocks that carried an *EcR-B1* mutant allele opposite a balancer chromosome and two copies of the relevant construct on isogenic third chromosomes.

A detailed diagram and explanation of the crossing scheme can be found in figure III–10. Using this crossing scheme, the following 3 stocks were created: *EcR*^{W53st}/*CyO*, *y*⁺; P[*Ddc-lacZ*]SH/ P[*Ddc-lacZ*]SH and *EcR*^{W53st}/*CyO*, *y*⁺; P[*Ddc-lacZ*]BH/ P[*Ddc-lacZ*]BH and *EcR*^{W53st}/*CyO*, *y*⁺; P[*Ddc-lacZ*]RH/ P[*Ddc-lacZ*]RH.

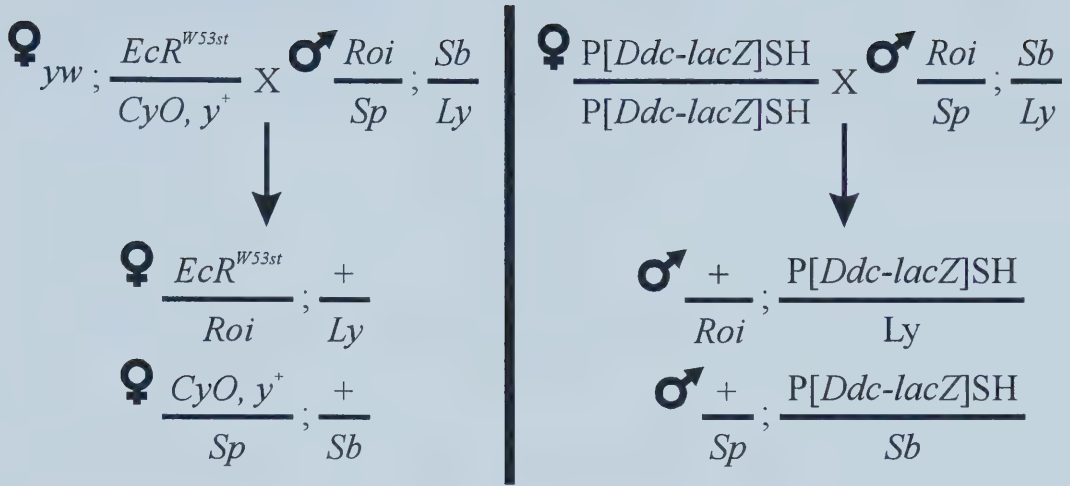
The stocks carrying the *EcR*^{W53st} allele and the reporter constructs are crossed to the *EcR*^{M554fs} null mutation to generate mutant organisms.

In a fashion similar to the stocks used for the RT-PCR assay (see above), these three newly generated stocks were crossed with the *EcR*^{M554fs} null mutation to generate

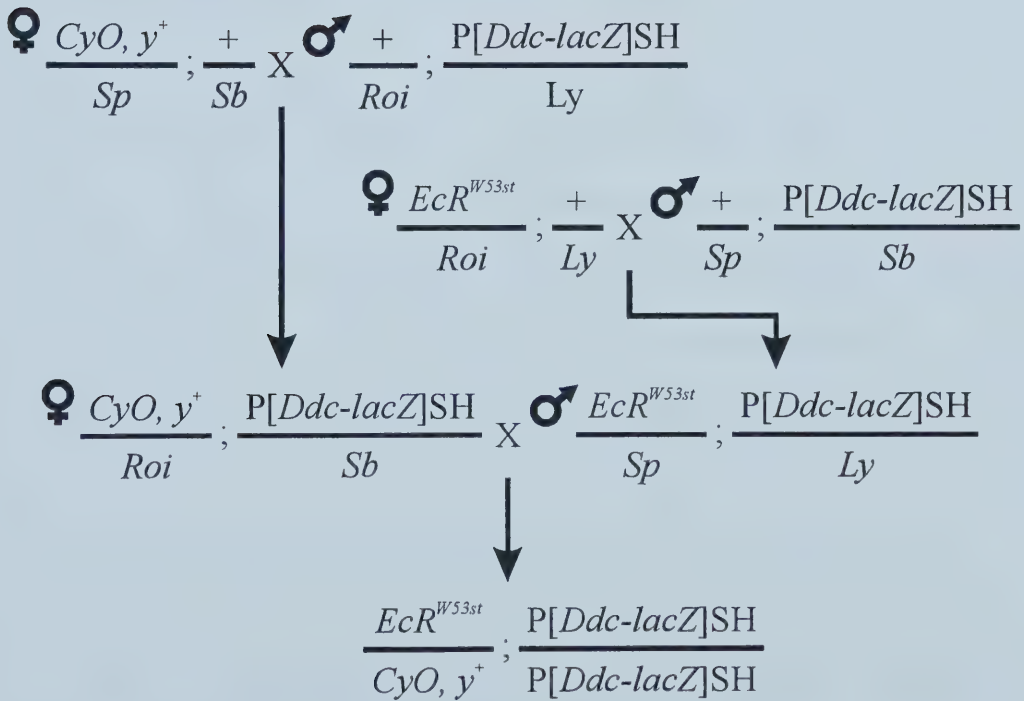
produce male offspring with a EcR^{W53st}/Sp ; $P[Ddc-lacZ]SH/Ly$ genotype. These individuals were isolated by selecting for the Sp and Ly phenotypes and against the Roi and Sb phenotypes. Lastly, the $CyO, y^+/Roi$; $P[Ddc-lacZ]SH/Sb$ virgin females generated above were crossed to the EcR^{W53st}/Sp ; $P[Ddc-lacZ]SH/Ly$ males generated above to produce offspring with a $EcR^{W53st}/CyO, y^+$; $P[Ddc-lacZ]SH/P[Ddc-lacZ]SH$ genotype. These organisms were isolated by selecting for the Cy phenotype and against the Roi , Sp , Sb , and Ly phenotypes. These organisms were then intercrossed to generate a true breeding line. Identical crosses were performed, save for the $P[Ddc-lacZ]$ construct used, to generate the $EcR^{W53st}/CyO, y^+$; $P[Ddc-lacZ]BH/P[Ddc-lacZ]BH$ and the $EcR^{W53st}/CyO, y^+$; $P[Ddc-lacZ]RH/P[Ddc-lacZ]RH$ lines. The presence of the EcR^{W53st} mutation in these new stocks was confirmed by performing a test cross with $yw; EcR^{M554fs}/CyO, y^+$ organisms, which generated offspring with an $EcR-BI$ mutant phenotype (see Fig. III-12).

Figure III–10 Diagram of the crossing scheme used to combine the *EcR^{W53st}* mutant with the P[*Ddc-lacZ*] reporter constructs. The construction of the *EcR^{W53st}/CyO, y⁺; P[Ddc-lacZ]SH/ P[Ddc-lacZ]SH* genotype is shown as an example. (A) Virgin females with a *yw; EcR^{W53st}/CyO, y⁺* genotype were crossed to *Roi/Sp; Sb/Ly* males. The *EcR^{W53st}/Roi, +/Ly* female offspring were isolated by selecting for the *Roi* and *Ly* phenotypes and against the *Cy*, *Sp*, and *Sb* phenotypes. Similarly, the *CyO, y⁺/Sp; +/Sb* females were isolated by selecting for the *Cy*, *Sp*, and *Sb* phenotypes and against the *Roi* and *Ly* phenotypes. At the same time virgin females with a P[*Ddc-lacZ*]SH/ P[*Ddc-lacZ*]SH genotype were crossed to *Roi/Sp; Sb/Ly* males. The *+/Roi, P[Ddc-lacZ]SH/Ly* male offspring were isolated by selecting for the *Roi* and *Ly* phenotypes and against the *Sp* and *Sb* phenotypes. Similarly, the the *+/Sp; P[Ddc-lacZ]SH/Sb* males were isolated by selecting for the *Sp* and *Sb* phenotypes and against the *Roi* and *Ly* phenotypes. (B) The *CyO, y⁺/Sp; +/Sb* virgin females generated above were then crossed to the *+/Roi; P[Ddc-lacZ]SH/Ly* males generated above to produce female offspring with a *CyO, y⁺/Roi; P[Ddc-lacZ]SH/Sb* genotype. These individuals were isolated by selecting for the *Cy*, *Roi*, and *Sb* phenotypes and against the *Sp* and *Ly* phenotypes. Similarly, the *EcR^{W53st}/Roi; +/Ly* virgin females generated above were then crossed to the *+/Sp; P[Ddc-lacZ]SH/Sb* males generated above to

A)



B)



EcR-B1 mutant and wild-type offspring (Figure III-11). The progeny were sexed and male organisms were staged in accordance with Andres and Thummel (1994). Mutant individuals were separated from wild-type individuals on the basis of their *yellow* phenotype.

The EcR-B1 protein plays a role in the precocious *Ddc* expression seen in constructs lacking the silencer region.

The offspring of crosses between the *EcR*^{M554fs} stock and the lines carrying the P[*Ddc-lacZ*]SH, P[*Ddc-lacZ*]BH, and P[*Ddc-lacZ*]RH constructs were analyzed at the early wandering stage of the third instar (Figure III-12). The organisms carrying the P[*Ddc-lacZ*]SH construct showed no precocious expression in either an *EcR-B1* mutant or a wild-type background (A, B). This is consistent with the results of Chen *et al.* (2002) and was to be expected given that the P[*Ddc-lacZ*]SH construct possesses the region harbouring the silencing element(s). Furthermore, both the P[*Ddc-lacZ*]BH and P[*Ddc-lacZ*]RH constructs showed precocious expression when placed in a wild-type background (D, F). Again, this is consistent with the evidence of Chen *et al.* (2002) and was to be expected given that these constructs lacked the silencer element(s). However, when these constructs were placed in an *EcR-B1* mutant it dramatically altered the results. Both the P[*Ddc-lacZ*]BH and P[*Ddc-lacZ*]RH constructs showed a complete loss of this precocious expression (C, E), suggesting that the putative silencer molecule(s) work to antagonize signaling by the ecdysone-EcR-B1 complex in early to mid third instar larvae.

Figure III-11 Generation of *EcR-B1* mutant organisms that carry the P[*Ddc-LacZ*]SH, P[*Ddc-LacZ*]BH, and P[*Ddc-LacZ*]RH constructs. In order to study the effect that the absence of EcR-B1 protein has on the activity of the P[*Ddc-LacZ*] constructs, the cross diagrammed in the figure (for P[*Ddc-lacZ*]SH) was carried out. The lines carrying the P[*Ddc-lacZ*]BH construct and the P[*Ddc-lacZ*]RH construct were crossed in an identical fashion. Male *EcR-B1* mutant organisms (*yellow*⁻ phenotype) and their wild-type siblings (*yellow*⁺ phenotype) were identified as described in Materials and Methods.

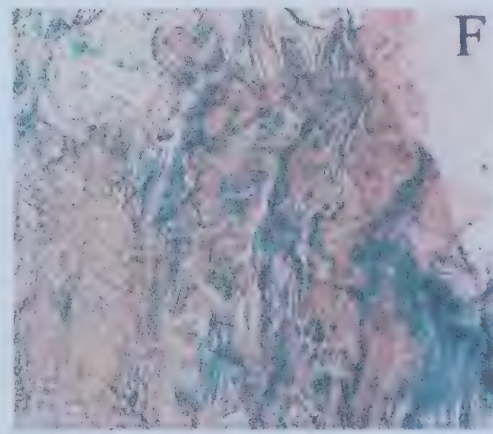
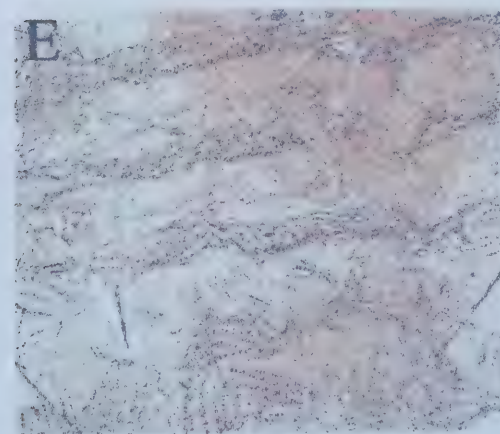
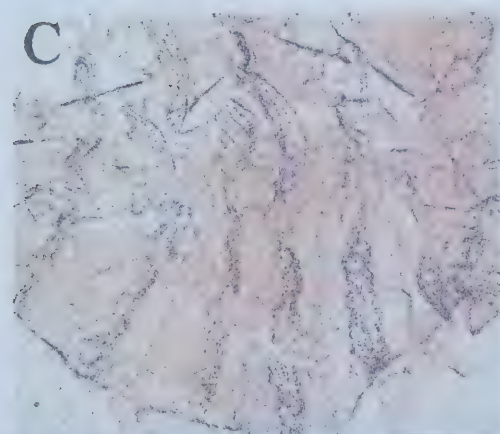
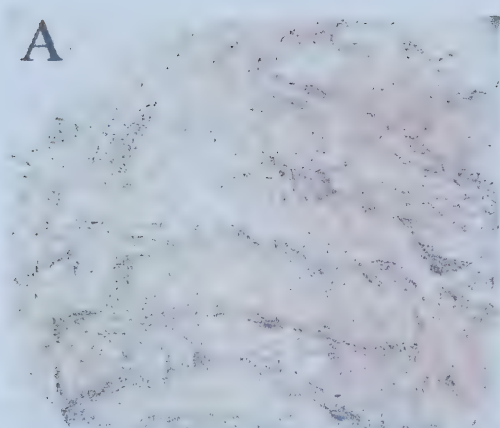
$$\text{♀ } \frac{yw}{Y}; \frac{EcR^{M554fs}}{CyO, y^+} \times \text{♂ } \frac{yw}{yw}; \frac{EcR^{W53st}}{CyO, y^+}; \frac{P[Ddc-lacZ]SH}{P[Ddc-lacZ]SH}$$



$$\text{♂ } \frac{yw}{Y}; \frac{EcR^{W53st}}{EcR^{M554fs}}; \frac{P[Ddc-lacZ]SH}{+} \quad \left. \vphantom{\frac{yw}{Y}} \right] \text{yellow phenotype}$$

$$\begin{array}{l} \text{♂ } \frac{yw}{Y}; \frac{EcR^{W53st}}{CyO, y^+}; \frac{P[Ddc-lacZ]SH}{+} \\ \text{♂ } \frac{yw}{Y}; \frac{EcR^{M554fs}}{CyO, y^+}; \frac{P[Ddc-lacZ]SH}{+} \end{array} \quad \left. \vphantom{\frac{yw}{Y}} \right] \text{yellow}^+ \text{ phenotype}$$

Figure III–12 Expression patterns of the P[*Ddc-lacZ*] constructs in the epidermis of *EcR^{W53st}* and wild-type wandering third instar larvae. The β -galactosidase activity shown is that generated by the P[*Ddc-lacZ*]SH (panels A, B), P[*Ddc-lacZ*]BH (C, D), and P[*Ddc-lacZ*]RH (E, F) constructs in the epidermis of *EcR^{W53st}* mutant (A, C, E) and wild-type (B, D, F) wandering third instar larvae.



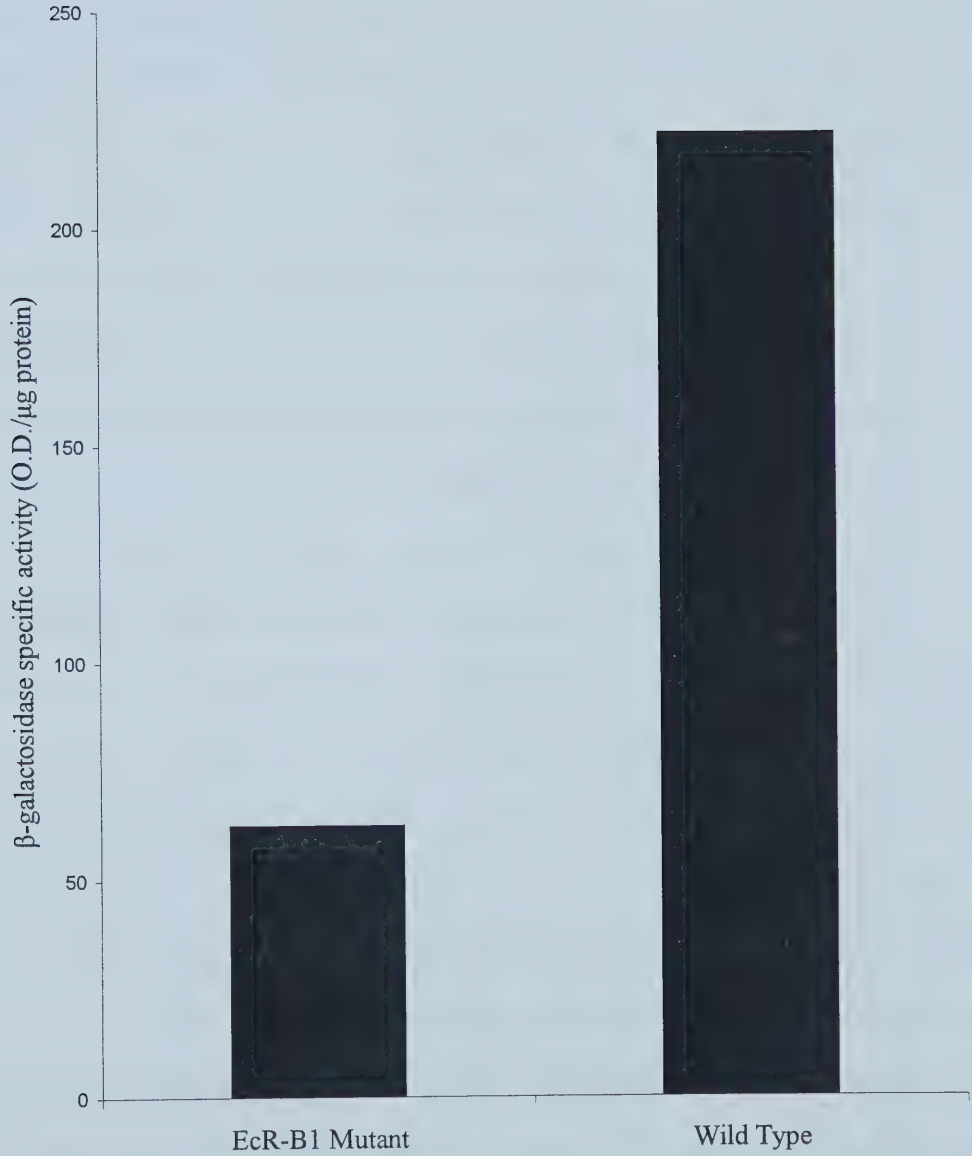
The P[*Ddc-lacZ*]SH construct shows severely reduced levels of expression in an *EcR-B1* mutant background at pupariation.

The P[*Ddc-lacZ*]SH construct has been shown to generate an almost 18-fold increase in β -galactosidase activity at pupariation in wild-type organisms (Chen *et al.*, 2002). By comparing, side by side, the levels of β -galactosidase activity generated by this construct in *EcR-B1* and wild-type backgrounds, it is possible to confirm the results of the RT-PCR assay. For the purpose of doing this, *yw; EcRW53st/ EcRM554fs; P[*Ddc-lacZ*]SH/+* males and wild-type sibling males segregating in the same cross (see Fig. III-11) were collected as white prepupae. Crude protein extracts were prepared from each pool of organisms and assayed for β -galactosidase activity (see Materials and Methods). The results show that in *EcR-B1* mutant organisms, the P[*Ddc-lacZ*]SH construct generates only just over 28% of the activity of the same construct in a wild-type organism (Fig. III-13).

An *in vitro* analysis of *Ddc* regulation in a cell culture system

The final portion of this thesis was aimed at developing a better understanding of those regions of the *Ddc* promoter critical for the gene's proper expression. We hoped that this could be done using an *in vitro* system that relied upon *Drosophila melanogaster* cell lines. By utilizing a cell-culture system, it is possible to more rapidly assay the functional significance of any experimental alterations made to the promoter of the *Ddc* gene than is possible using transgenics. In addition, it is possible for the experimenter to control for the presence and/or absence of the various EcR

Figure III-13 Activity of the P[*Ddc-lacZ*]SH construct in *EcR*^{W53st}/*EcR*^{M554fs} (indicated as mutant) and wild-type (either *EcR*^{W53st}/*CyO*, *y*⁺ or *EcR*^{M554fs}/*CyO*, *y*⁺ genotypes; indicated as wild type) organisms. The diagram shows the average level of β -galactosidase activity, measured in O.D. units per μ g of total protein assayed, seen in *EcR*^{W53st} mutant and wild-type organisms at the onset of pupariation. The values shown in the diagram represent the average of two assays done on each pool of three independently collected pools of organisms.



protein isoforms. This allows for inferences to be made in regard to the role of each EcR isoform in *Ddc* expression. However, a number of conditions must be met prior to this being a viable way of studying *Ddc* expression. First, fragments of the regulatory regions of the *Ddc* gene must be fused to the coding region of a reporter gene in a plasmid that can be transfected into *Drosophila* cells. In the case of this study, the constructs were derivatives of the lacZ reporter vector P{CaSpeR-AUG- β -gal} (Thummel *et al.*, 1998) that contained fragments of the 5' portion of the *Ddc* gene (Li Chen *et al.*, 2002; gift of Li Chen, see Materials and Methods).

Furthermore, because these constructs were used to generate transgenic flies, the potential exists to compare *in vivo* and *in vitro* results. Second, it is necessary to use a line of *Drosophila* cells that does not express the ecdysone receptor to control for the role of the ecdysone-receptor complex in *Ddc* induction. This is important because most common *Drosophila* cell lines express a poorly defined set of EcR isoforms and will generate a response to ecdysone even in the absence of a transient source of the receptor, thus creating uninterpretable results. The use of a cell line that largely doesn't express the receptor, which in this case was the L57-3-11 *Drosophila* cell line (Cherbas and Cherbas, 1997), prevents this interference by eliminating the vast majority of endogenous EcR activity in the cell line. Third, an exogenous source of the EcR isoforms is needed to both restore ecdysone responsiveness to the cell line and study the role that each isoform plays in *Ddc* expression. In this study, the exogenous EcR was supplied by transfecting the L57-3-11 cell line with plasmids that express the A, B1, or B2 isoforms (gift of Peter Cherbas, see Materials and Methods).

The L57-3-11 cell line responds to ecdysone following transfection.

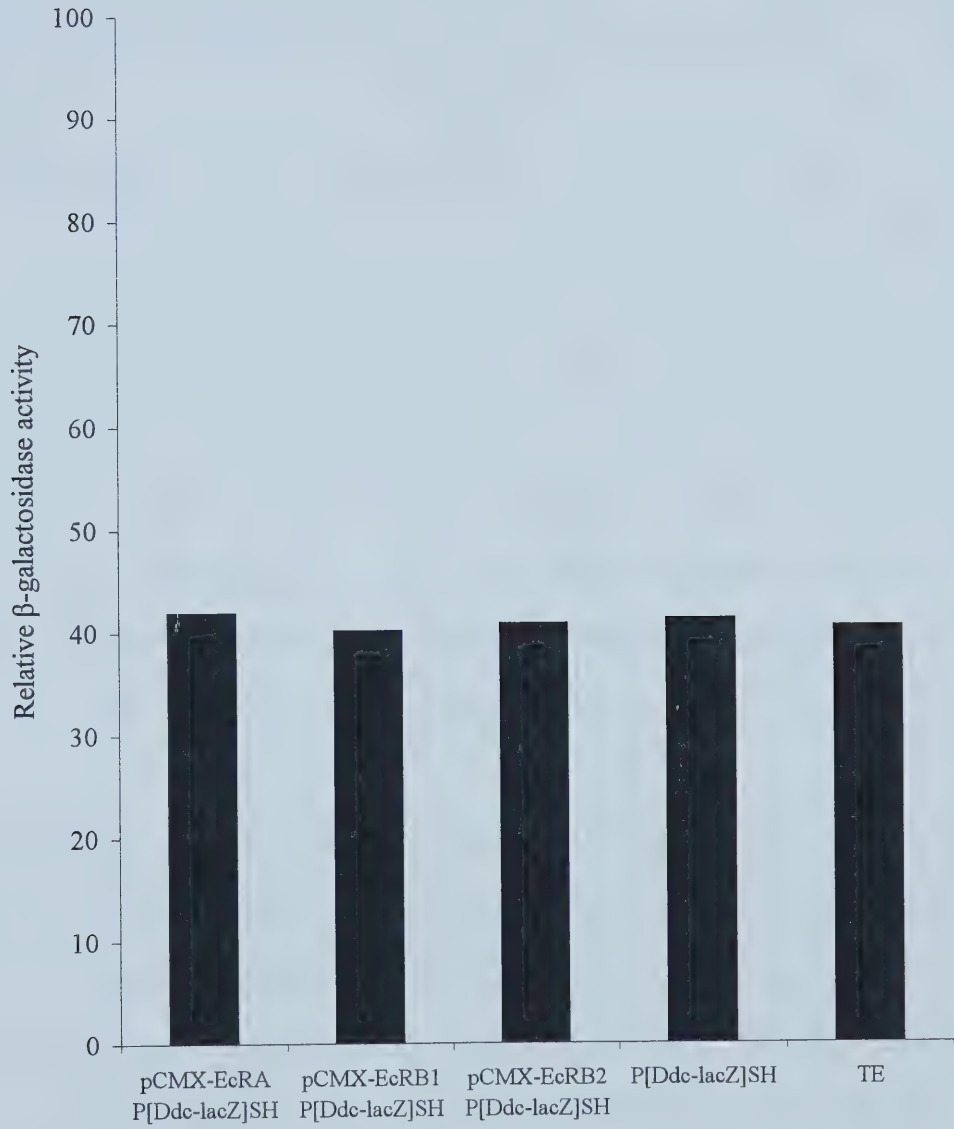
Before proceeding with an attempt to induce *Ddc* expression with ecdysone, it was necessary to confirm that the L57-3-11 cells were responsive to the administration of ecdysone following transfection with the EcR expression vectors. To do so, the L57-3-11 cells were co-transfected with pCMX-EcRB1, the vector that expresses the EcR-B1 protein, and pPal1-SX -188-cc-luc, a vector that carries a luciferase reporter driven by a tandem array of seven EcREs. Aliquots of the transfected cells were treated with ecdysone or ethanol for 24 hours and luciferase activity was then assayed as described in Materials and Methods. The average level of induction in cells treated with ecdysone was found to be over 46 times that of the ethanol treated aliquots of cells (data not shown). This is consistent with previous results that showed approximately 40 times the level of luciferase activity in ecdysone-treated samples versus control samples (Lucy Cherbas, pers. comm.) and indicates that transfection with the EcR expression vectors restores ecdysone responsiveness to the L57-3-11 cells.

The *Ddc-lacZ* constructs are not induced when co-transfected with the EcR expression vectors.

The first construct to be tested in the cell culture system was P[*Ddc-lacZ*]SH. This construct consists of a *Ddc* fragment extending from a *Sca* I site in the 5'-flanking region of the gene to a *Hpa* I site in the 3' region of the first exon of the gene (Fig. III-9). This fragment of the *Ddc* gene carries the EcRE, BR-C response element(s), and the silencer element(s) (detailed above) and behaves in a wild-type fashion *in vivo* (Li Chen, pers. comm.). It was necessary to collect cells from five separate

flasks for the transfection experiment. Cells from the first, second, and third flasks were subsequently transfected with P[*Ddc-lacZ*]SH and pCMX-EcRA, P[*Ddc-lacZ*]SH and pCMX-EcRB1, and P[*Ddc-lacZ*]SH and pCMX-EcRB2, respectively. The fourth flask of cells was transfected with P[*Ddc-lacZ*]SH alone and the fifth flask of cells was mock transfected to provide the appropriate control. Following treatment with either ecdysone or the ethanol solvent, the β -galactosidase activity in each aliquot of cells was assayed a total of three times. The transfections were then repeated to generate a duplicate set of results. The results show that the mock-transfected cells produced nearly a 42-fold increase in β -galactosidase activity when treated with ecdysone (Fig. III-14). This increase in activity is the result of ecdysone-induced expression of the *Drosophila* β -galactosidase gene (Best-Belpomme *et al.*, 1978) that is mediated by the residual EcR-A protein expressed by the L57-3-11 cells (Lucy Cherbas, pers. comm.). The figure also shows cells transfected with P[*Ddc-lacZ*]SH alone produced a similar increase in β -galactosidase activity when treated with ecdysone, indicating that in the absence of exogenous EcR, the reporter construct fails to produce a measurable level of β -galactosidase. However, the cells transfected with combinations of P[*Ddc-lacZ*]SH and pCMX-EcRA, pCMX-EcRB1, or pCMX-EcRB2 also only generated roughly 40-fold increases in β -galactosidase activity when treated with ecdysone. These levels of induction are almost identical to those of the control samples, indicating that the exogenously supplied EcR protein isoforms were incapable of generating a measurable level of expression from the P[*Ddc-lacZ*]SH construct.

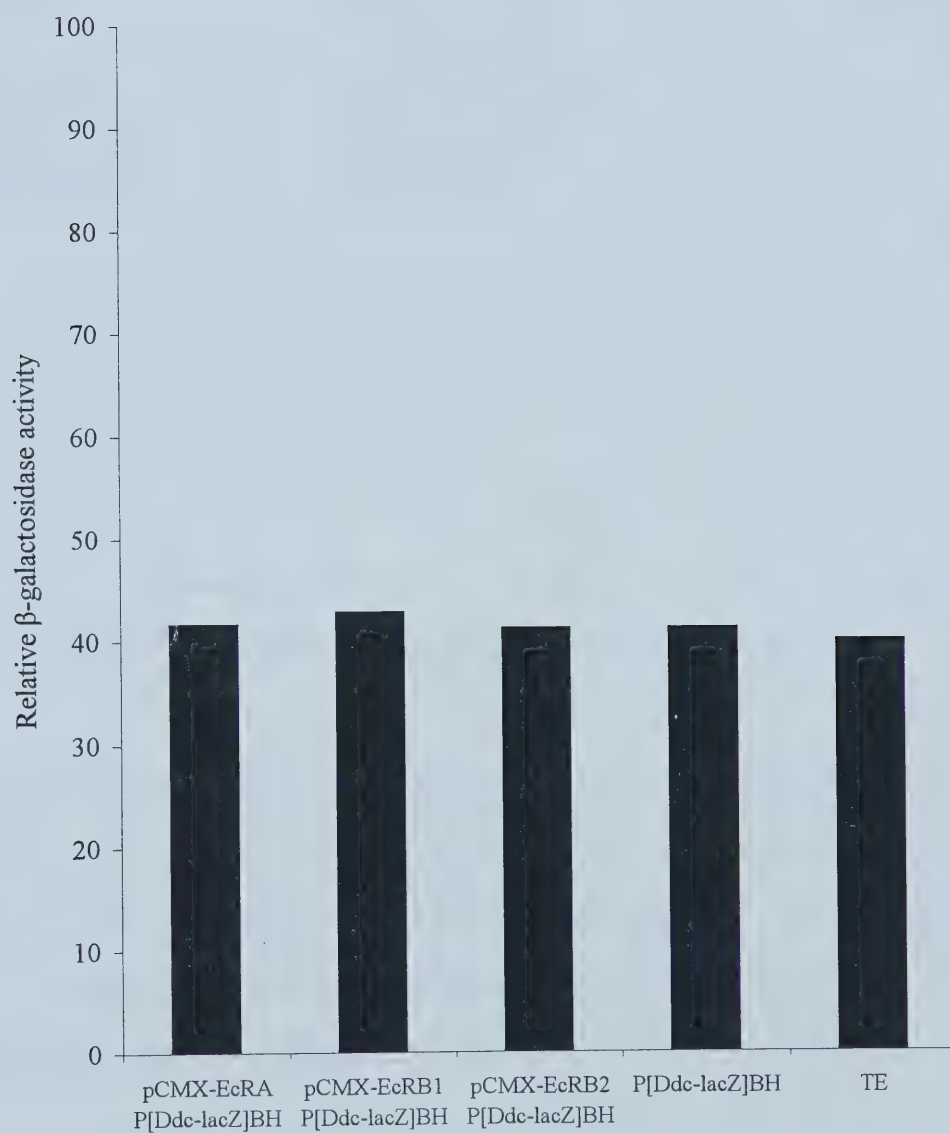
Figure III-14 β -galactosidase activity in L57-3-11 cells transfected with the P[*Ddc-lacZ*]SH construct. The cells were transfected with plasmid DNAs and aliquots were either treated with ecdysone or ethanol. Cell extracts were then assayed for β -galactosidase activity. The results are shown as a ratio of the β -galactosidase activity in ecdysone treated cells compared to ethanol treated cells, with the plasmid DNAs used for each transfection being indicated on the abscissa. The values shown are the average of two separate transfection experiments, with the cell extracts from each transfection being assayed in triplicate.



The next construct to be tested was P[*Ddc-lacZ*]BH. This construct consists of a *Ddc* fragment extending from a *Bsm* I site in the 5'-flanking region of the gene to a *Hpa* I site in the 3' region of the first exon of the gene (Fig. III-9). This fragment possesses both the BR-C response element(s) and the EcRE, but lacks the suppressor element(s). Again, five flasks of cells were collected and either mock transfected, transfected with P[*Ddc-lacZ*]BH alone, or transfected with P[*Ddc-lacZ*]BH and pCMX-EcRA, P[*Ddc-lacZ*]BH and pCMX-EcRB1, or P[*Ddc-lacZ*]BH and pCMX-EcRB2. The results, detailed in figure III-15, show very similar levels of induction following treatment with ecdysone, regardless of whether the cells were mock transfected, transfected with P[*Ddc-lacZ*]BH alone, or transfected with a combination of P[*Ddc-lacZ*]BH and pCMX-EcRA, pCMX-EcRB1, or pCMX-EcRB2. Again, these results suggest that the exogenous EcR proteins fail to generate measurable expression from the P[*Ddc-lacZ*]BH construct and that the β -galactosidase activity can be wholly attributed to the *Drosophila* β -galactosidase gene.

The final construct to be tested was P[*Ddc-lacZ*]RH. This construct consists of a *Ddc* fragment extending from an *EcoR* V site just upstream of the first exon of the gene to a *Hpa* I site at the 3' end of the first exon (Fig. III-9). This fragment extends just upstream of the EcRE, but lacks the other regulatory elements. As with the other sets of transfections, flasks of cells were collected and either mock transfected, transfected with P[*Ddc-lacZ*]RH alone, or transfected with a combination of P[*Ddc-lacZ*]RH and pCMX-EcRA, pCMX-EcRB1, or pCMX-EcRB2. As with the other sets of transfections, the presence of the exogenous EcR protein isoforms failed to generate a

Figure III-15 β -galactosidase activity in L57-3-11 cells transfected with the P[*Ddc-lacZ*]BH construct. The cells were transfected with plasmid DNAs and aliquots were either treated with ecdysone or ethanol. Cell extracts were then assayed for β -galactosidase activity. The results are shown as a ratio of the β -galactosidase activity in ecdysone treated cells compared to ethanol treated cells, with the plasmid DNAs used for each transfection being indicated on the abscissa. The values shown are the average of two separate transfection experiments, with the cell extracts from each transfection being assayed in triplicate.



significant increase in β -galactosidase activity when compared to the control transfections (Fig. III-16). This indicated that again no measurable expression was being generated from the P[*Ddc-lacZ*]RH construct.

The construction of vectors that express the BR-C protein isoforms.

The final stage of the *in vitro* cell culture assays involved testing the ability of the *BR-C* gene products to generate expression from the *Ddc-lacZ* constructs. Before the role of *BR-C* could be studied in the cell culture system, it was necessary to create plasmid constructs that were capable of providing an exogenous source of the four BR-C protein isoforms. The constructs generated for this purpose were derivatives of the pPac-PL vector (Krasnow *et al.*, 1989) and contained cDNA clones with the coding regions for BR-C Z1 (dm527), BR-C Z2 (CD5), BR-C Z3 (dm797), and BR-C Z4 (28-I) proteins (Dibello *et al.* 1991; Bayer *et al.*, 1996; see Fig. III-17).

The BR-C Z1 expression vector, called pPac-PL(Z1), was created by inserting the dm527 cDNA clone into the pPac-PL expression vector. This was done by first removing the dm527 cDNA from its Bluescribe vector by digestion with *EcoR* I. The cDNA clone was then blunt ended and ligated to the blunt ends of an *EcoR* V-digested and dephosphorylated pPac-PL vector.

The BR-C Z2 expression vector, called pPac-PL(Z2), was created by inserting the CD5 cDNA clone into the pPac-PL expression vector. This was done by first releasing the CD5 cDNA clone from its Bluescript vector as a *Kpn* I-*Sma* I fragment

Figure III-16 β -galactosidase activity in L57-3-11 cells transfected with the P[*Ddc-lacZ*]RH construct. The cells were transfected with plasmid DNAs and aliquots were either treated with ecdysone or ethanol. Cell extracts were then assayed for β -galactosidase activity. The results are shown as a ratio of the β -galactosidase activity in ecdysone treated cells compared to ethanol treated cells, with the plasmid DNAs used for each transfection being indicated on the abscissa. The values shown are the average of two separate transfection experiments, with the cell extracts from each transfection being assayed in triplicate.

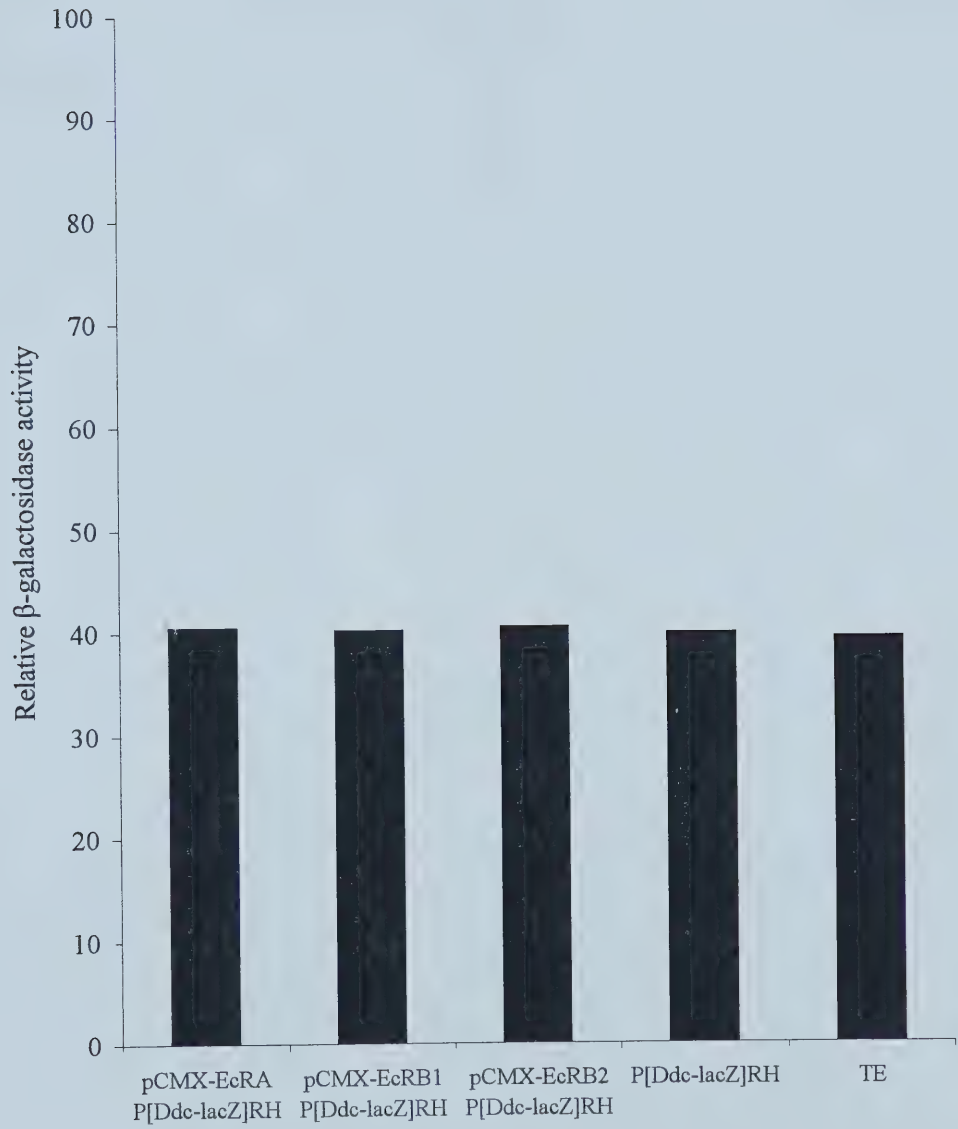
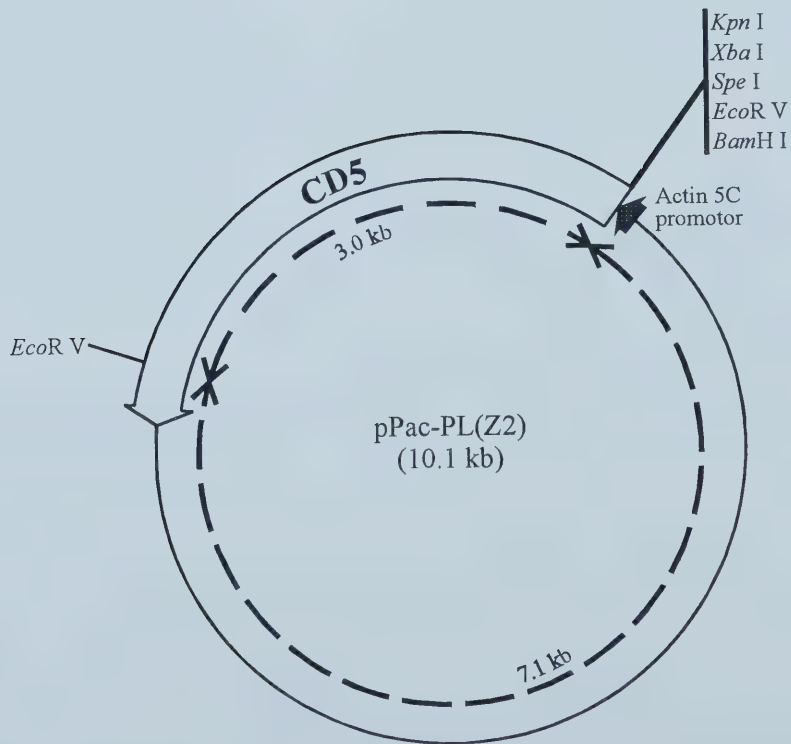
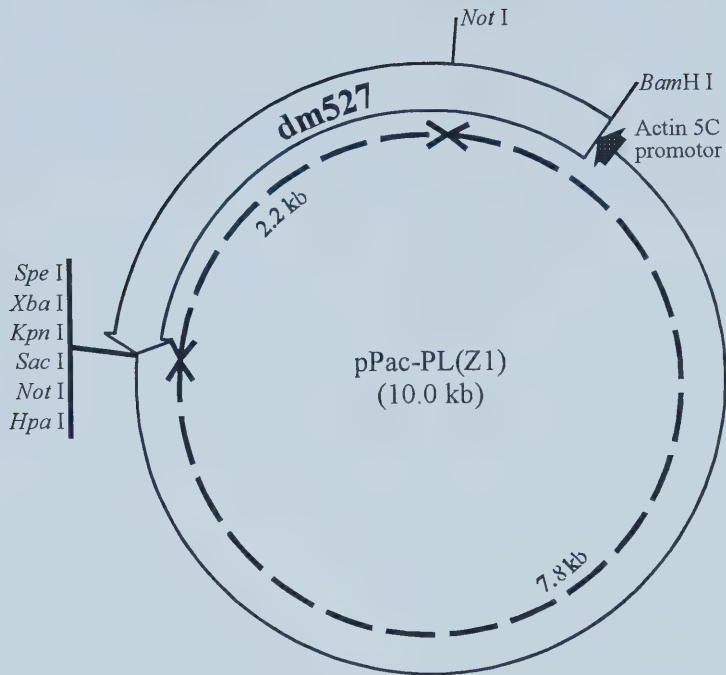
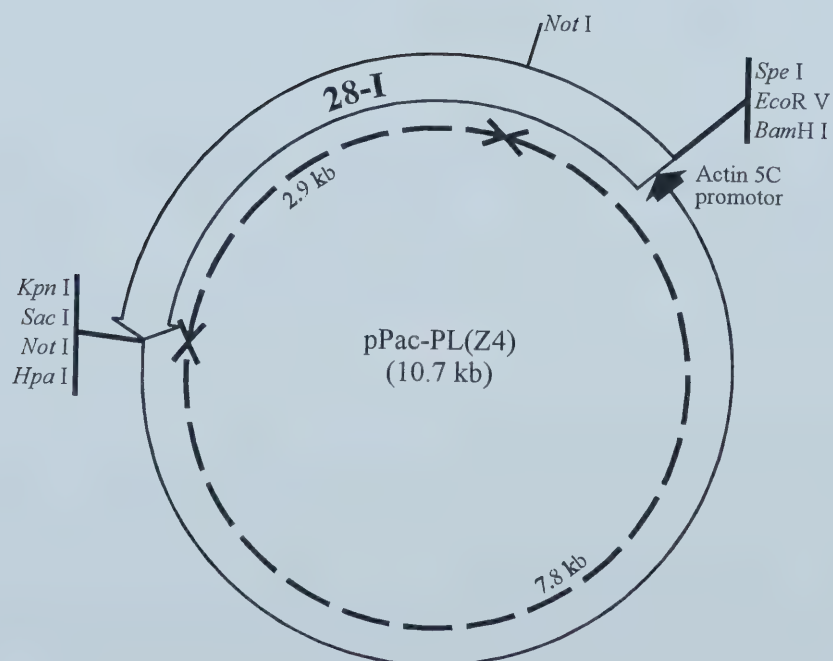
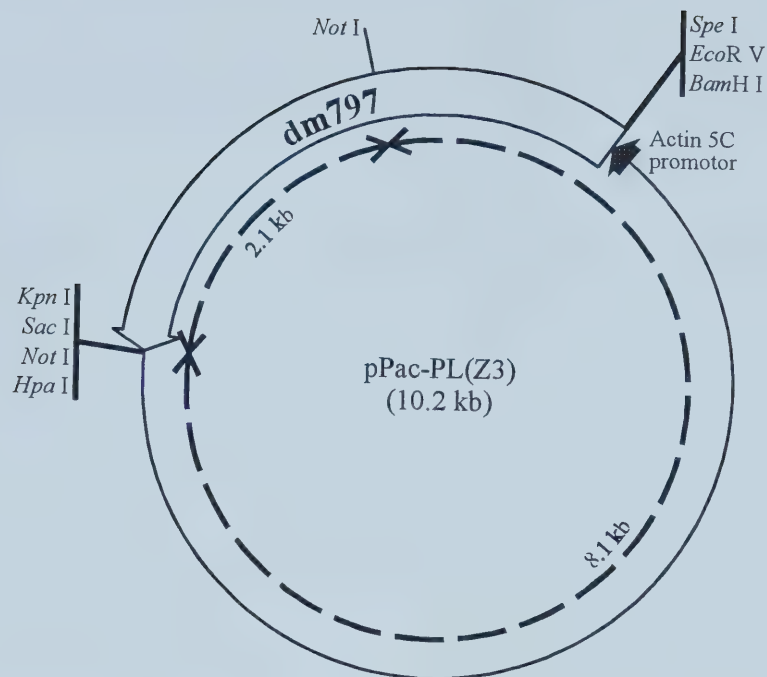


Figure III-17 Schematic diagram of the BR-C expression vectors. All four plasmid constructs are derivatives of pPac-PL (Krasnow *et al.*, 1989). The pPac-PL(Z1) vector contains the dm527 cDNA clone, which encodes the BR-C Z1 protein (DiBello *et al.*, 1991). The pPac-PL(Z2) vector contains the CD5 cDNA clone, which encodes the BR-C Z2 protein (DiBello *et al.*, 1991). The pPac-PL(Z3) vector contains the dm797 cDNA clone, which encodes the BR-C Z3 protein (DiBello *et al.*, 1991). The pPac-PL(Z4) vector contains the dm527 cDNA clone, which encodes the BR-C Z4 protein (Bayer *et al.*, 1996). The dashed curves on the inside of each plasmid indicate the size and location of the restriction fragments used to determine the identity of each construct (see Results).





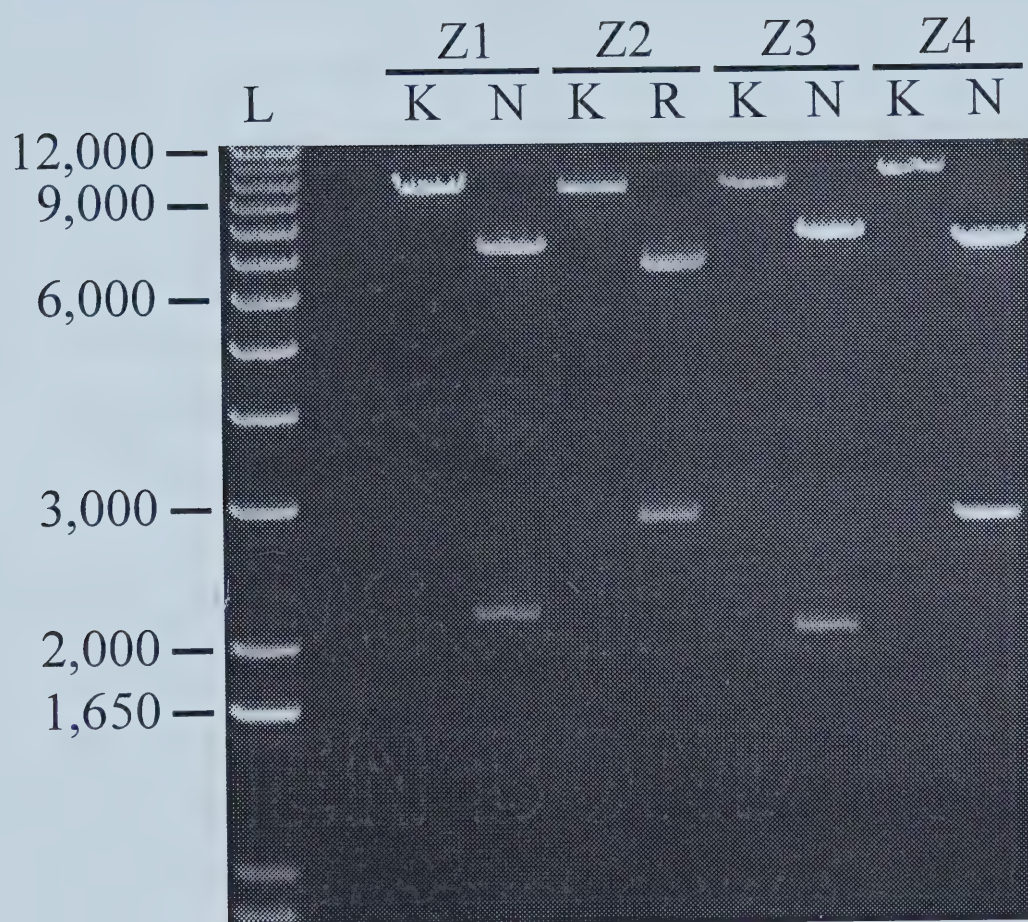
and double digesting the pPac-PL vector with *Kpn* I and *Hpa* I. The recombinant pPac-PL(Z2) vector was then formed by ligating the two DNA fragments, with the blunt *Sma* I end of the cDNA joining with the blunt *Hpa* I end of the pPac-PL vector.

The BR-C Z3 expression vector, called pPac-PL(Z3), was created by inserting the dm797 cDNA clone into the pPac-PL expression vector. This was done by first releasing the dm797 cDNA clone from its Bluescript II vector as a *Kpn* I-*Sma* I fragment and double digesting the pPac-PL vector with *Kpn* I and *EcoR* V. The recombinant pPac-PL(Z2) vector was then formed by ligating the two DNA fragments, with the blunt *Sma* I end of the cDNA joining with the blunt *EcoR* V end of the pPac-PL vector.

The BR-C Z4 expression vector, called pPac-PL(Z4), was created by inserting the 28-I cDNA clone into the pPac-PL expression vector. This was done by first releasing the 28-I cDNA clone from its Bluescript II vector as a *Kpn* I-*Spe* I fragment, while at the same time double digesting the pPac-PL vector with *Kpn* I and *Spe* I. The recombinant vector was then formed by directionally cloning the 28-I cDNA fragment into the digested pPac-PL vector.

The size of these recombinant molecules was then determined by restriction enzyme digestion. The four recombinant plasmids were converted into single linear molecules by digesting with *Kpn* I and analyzed by gel electrophoresis. As shown in figure III-18, the results are consistent with the expected sizes of the pPac-PL(Z1)

Figure III–18 Analysis of the BR-C expression vectors by restriction enzyme digestion. The BR-C expression vectors are indicated as follows: Z1 for pPac-PL(Z1), Z2 for pPac-PL(Z2), Z3 for pPac-PL(Z3), and Z4 for pPac-PL(Z4). The BR-C expression vectors were digested with *Kpn* I (K) and either *Not* I (N) or *EcoR* V (R) and the resulting fragments were separated by gel electrophoresis. The expected vector sizes and banding patterns are detailed in figure III–17. L, 1 Kb Plus DNA Ladder (Invitrogen) with representative band sizes indicated in base pairs.



plasmid (10.0 kb), pPac-PL(Z2) plasmid (10.1 kb), pPac-PL(Z3) plasmid (10.2 kb), and pPac-PL(Z4) plasmid (10.7 kb).

Next, the identity and orientation of the inserts in the pPac-PL vectors was confirmed by digesting pPac-PL(Z1) with *Not* I, pPac-PL(Z2) with *Eco*R V, pPac-PL(Z3) with *Not* I, and pPac-PL(Z4) with *Not* I. The restriction enzyme used to digest each construct was chosen because it would generate a specific set of bands only if the appropriate cDNA clone had been inserted into the pPac-PL vector in the correct orientation. The two sites where each restriction enzyme cuts its respective vector and the expected products of these digestions are shown in figure III–17. The results of the restriction digestions of the four expression vectors, shown in figure III–18, are consistent with the expected results.

Lastly, the results of the restriction analysis were corroborated using a PCR assay. The pPac-PL(Z1), pPac-PL(Z2), pPac-PL(Z3), and pPac-PL(Z4) vectors were added to PCR reaction mixtures containing primer pairs specific to the *BR-C Z1* cDNA, *BR-C Z2* cDNA, *BR-C Z3* cDNA, or *BR-C Z4* cDNA, respectively, and amplified as previously described (Hodgetts *et al.*, 1995). In addition, four control reactions were prepared that contained one of each of the four primer pairs and the pPac-PL parental vector. This was done to show that the pPac-PL vector was not acting as a template in any of the PCR reactions. The results, shown in figure III–19, are consistent with the expected product sizes of 777 bp for the *BR-C Z1*-specific primer pair, 320 bp for the *BR-C Z2*-specific primer pair, 784 bp for the *BR-C Z3*-specific primer pair, and 1082 bp for the *BR-C Z4*-specific primer pair (Hodgetts *et al.*, 1995).

Figure III–19 PCR analysis of the BR-C expression constructs. The BR-C

expression constructs are indicated as follows:

1: pPac-PL(Z1)

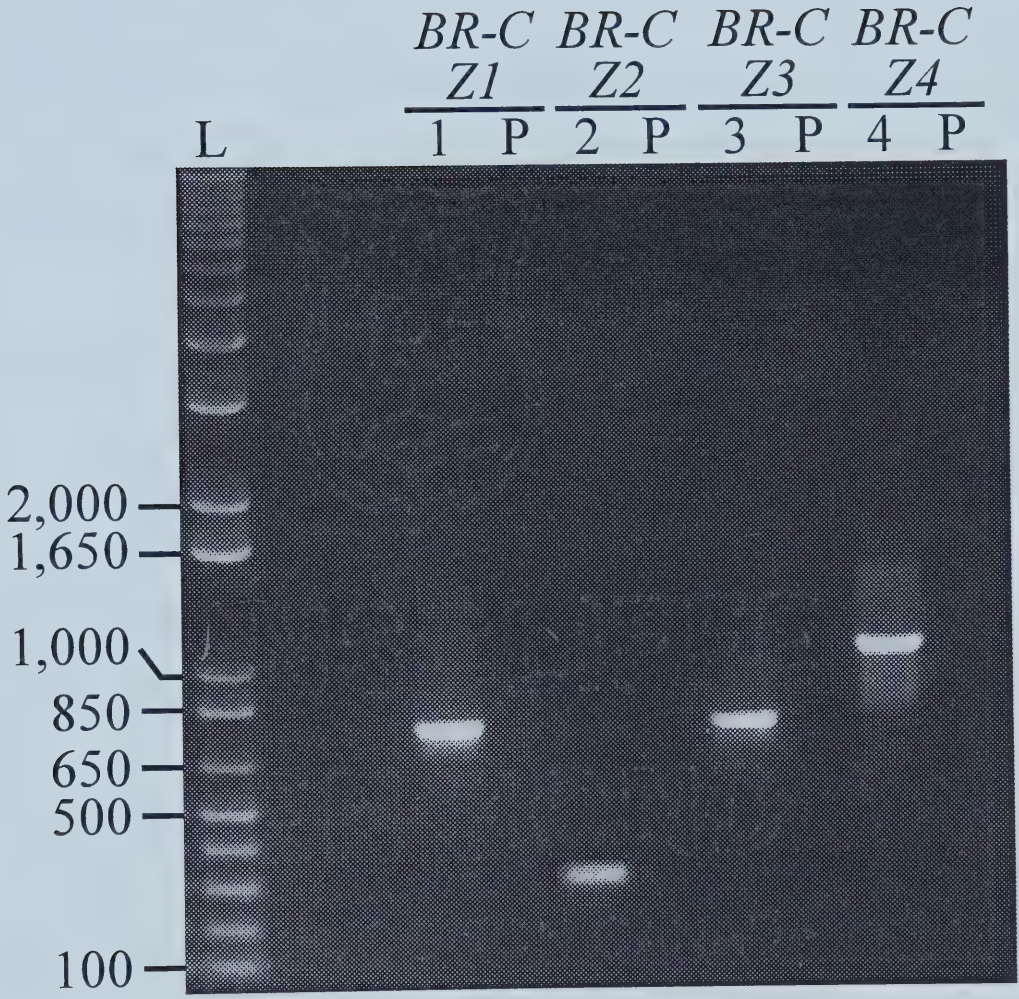
2: pPac-PL(Z2)

3: pPac-PL(Z3)

4: pPac-PL(Z4)

P: pPac-PL

The BR-C expression constructs were amplified as per Hodgetts *et al.* (1995) and then analyzed by gel electrophoresis. The specificity of the primer set used in each PCR reaction is indicated at the top of the figure. The expected sizes of the PCR products are 777 bp using the *BR-C Z1*-specific primers, 320 bp using the *BR-C Z2*-specific primers, 784 bp using the *BR-C Z3*-specific primers, and 1082 bp using the *BR-C Z4*-specific primers. L, 1 Kb Plus DNA Ladder (Invitrogen) with the sizes of representative bands indicated in base pairs.



The *Ddc-lacZ* constructs are not induced when co-transfected with the EcR and BR-C expression vectors.

It is possible that the lack of exogenous receptor-dependent induction of the *Ddc-lacZ* constructs seen above is due to inadequate levels of BR-C protein in the L57-3-11 cells. This hypothesis is quite plausible given that it has previously been shown that *BR-C* mutants can reduce *Ddc* expression to less than five per cent of wild-type levels (O’Keefe *et al.*, 1995). To test this hypothesis, expression from the P[*Ddc-lacZ*]SH construct was studied in the presence of exogenously supplied BR-C protein isoforms.

Previous evidence has shown that full induction of the *Ddc* gene at pupariation is highly dependent upon the BR-C Z2 protein isoform (O’Keefe *et al.*, 1995; Hodgetts *et al.*, 1995; Bayer *et al.* 1997). These data suggest that the BR-C Z2 isoform would be most likely to generate expression from one of *Ddc-lacZ* constructs and for this reason it was the first to be assayed. As with the original transfection experiments, three separate plates of cells were required to test the various combinations of constructs. The first flask of cells was transfected with P[*Ddc-lacZ*]SH, pPac-PL(Z2), and pCMX-EcRA. The second flask of cells was transfected with P[*Ddc-lacZ*]SH, pPac-PL(Z2), and pCMX-EcRB1. The third flask of cells was transfected with P[*Ddc-lacZ*]SH, pPac-PL(Z2), and pCMX-EcRB2. In addition, two flasks of cells were collected for use as controls. One flask of cells was transfected with P[*Ddc-lacZ*]SH alone and the other flask of cells was mock transfected. Following treatment with ecdysone or ethanol, the aliquots of cells from each transfection were

assayed for β -galactosidase activity. The results, displayed in figure III–20, showed an almost identical level of β -galactosidase activity amongst the five transfections. This indicates that all the β -galactosidase is due to expression from the endogenous *Drosophila* β -galactosidase gene and that the BR-C Z2 protein is incapable of generating expression from the P[*Ddc-lacZ*]SH construct.

Although less likely than BR-C Z2, the Z1, Z3, or Z4 isoforms of BR-C may be able to generate measurable amounts of expression from the *Ddc-lacZ* constructs. To test this hypothesis, the pPac-PL(Z1), pPac-PL(Z3), and pPac-PL(Z4) plasmids were used in a series of three separate transfection experiments, with each experiment designed to study the effect of one of these three BR-C isoforms. Each experiment involved transfecting three separate flasks of cells with the chosen BR-C expression vector, P[*Ddc-lacZ*]SH, and one of pCMX-EcRA, pCMX-EcRB1, or pCMX-EcRB2, in a fashion identical to that for pPac-PL(Z2) described above. In addition, each experiment included a flask of cells that was transfected with P[*Ddc-lacZ*]SH alone and a flask of cells that was mock transfected. The results indicate that neither exogenous BR-C Z1 (Fig. III–21), exogenous BR-C Z3 (Fig. III–22), nor exogenous BR-C Z4 (Fig. III–23) is capable of generating a significant increase in the amount of β -galactosidase when compared to the cells that were mock transfected. As with all the other transfection experiments performed in this thesis, the increased β -galactosidase activity seen in the cells following treatment with ecdysone is the result of expression from the endogenous *Drosophila* β -galactosidase gene.

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Figure III-20 The effect of exogenous BR-C Z2 protein on P[*Ddc-lacZ*]SH-generated β -galactosidase activity in L57-3-11 cells. The cells were transfected with plasmid DNAs and aliquots were either treated with ecdysone or ethanol. Cell extracts were then assayed for β -galactosidase activity. The results are shown as a ratio of the β -galactosidase activity in ecdysone treated cells compared to ethanol treated cells, with the plasmid DNAs used for each transfection being indicated on the abscissa. The values shown are the average of two separate transfection experiments, with the cell extracts from each transfection being assayed in triplicate.

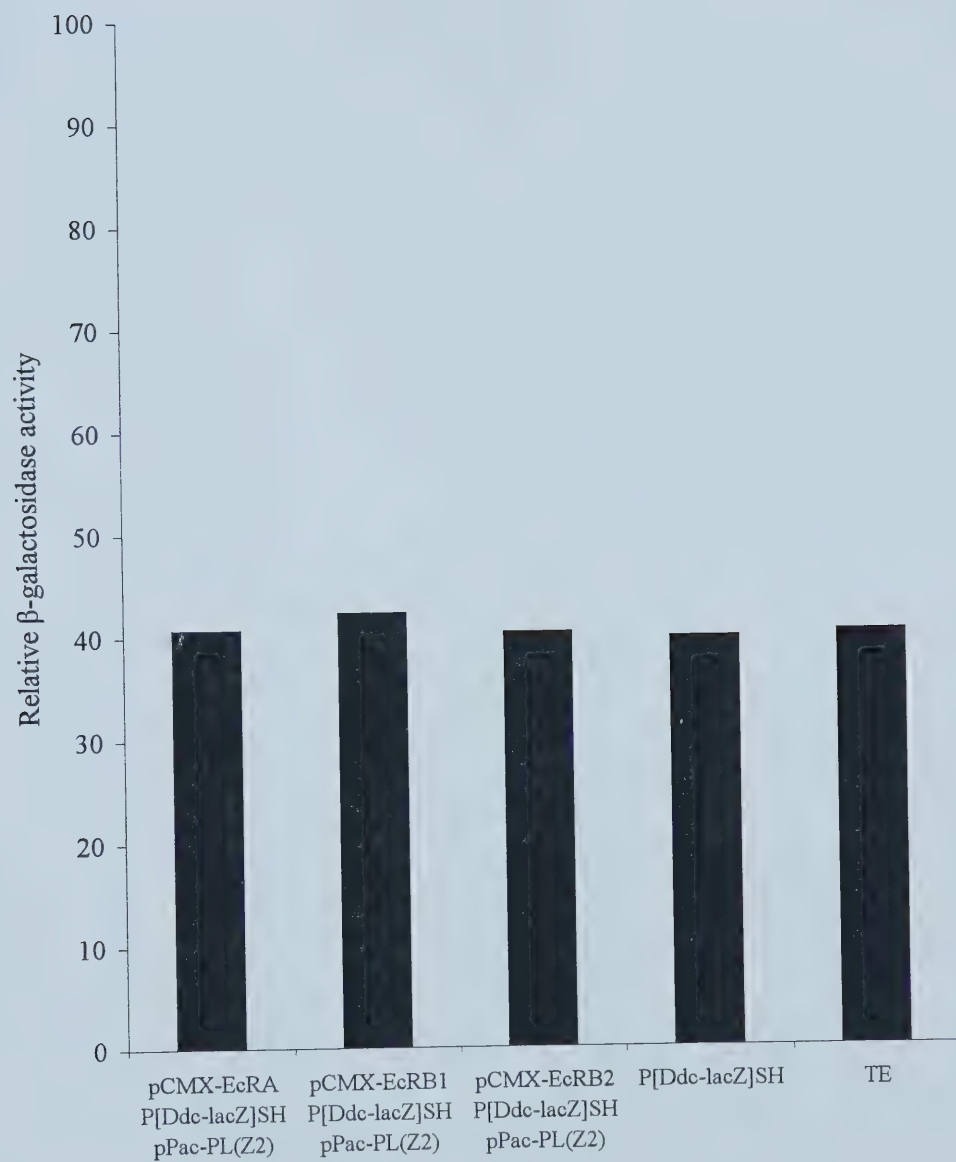
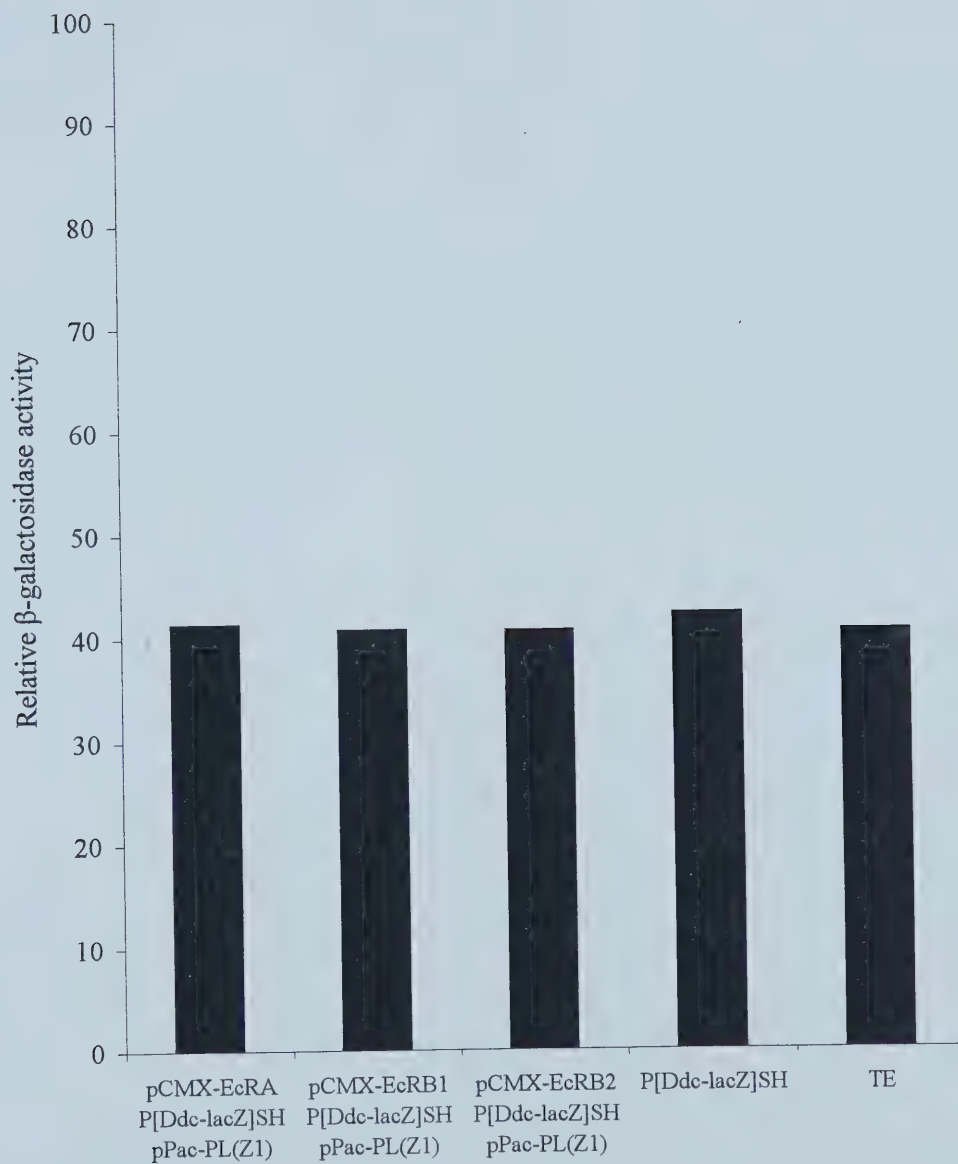


Figure III-21 The effect of exogenous BR-C Z1 protein on P[*Ddc-lacZ*]SH-generated β -galactosidase activity in L57-3-11 cells. The cells were transfected with plasmid DNAs and aliquots were either treated with ecdysone or ethanol. Cell extracts were then assayed for β -galactosidase activity. The results are shown as a ratio of the β -galactosidase activity in ecdysone treated cells compared to ethanol treated cells, with the plasmid DNAs used for each transfection being indicated on the abscissa. The values shown are the average of two separate transfection experiments, with the cell extracts from each transfection being assayed in triplicate.



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Figure III-22 The effect of exogenous BR-C Z3 protein on P[*Ddc-lacZ*]SH-generated β -galactosidase activity in L57-3-11 cells. The cells were transfected with plasmid DNAs and aliquots were either treated with ecdysone or ethanol. Cell extracts were then assayed for β -galactosidase activity. The results are shown as a ratio of the β -galactosidase activity in ecdysone treated cells compared to ethanol treated cells, with the plasmid DNAs used for each transfection being indicated on the abscissa. The values shown are the average of two separate transfection experiments, with the cell extracts from each transfection being assayed in triplicate.

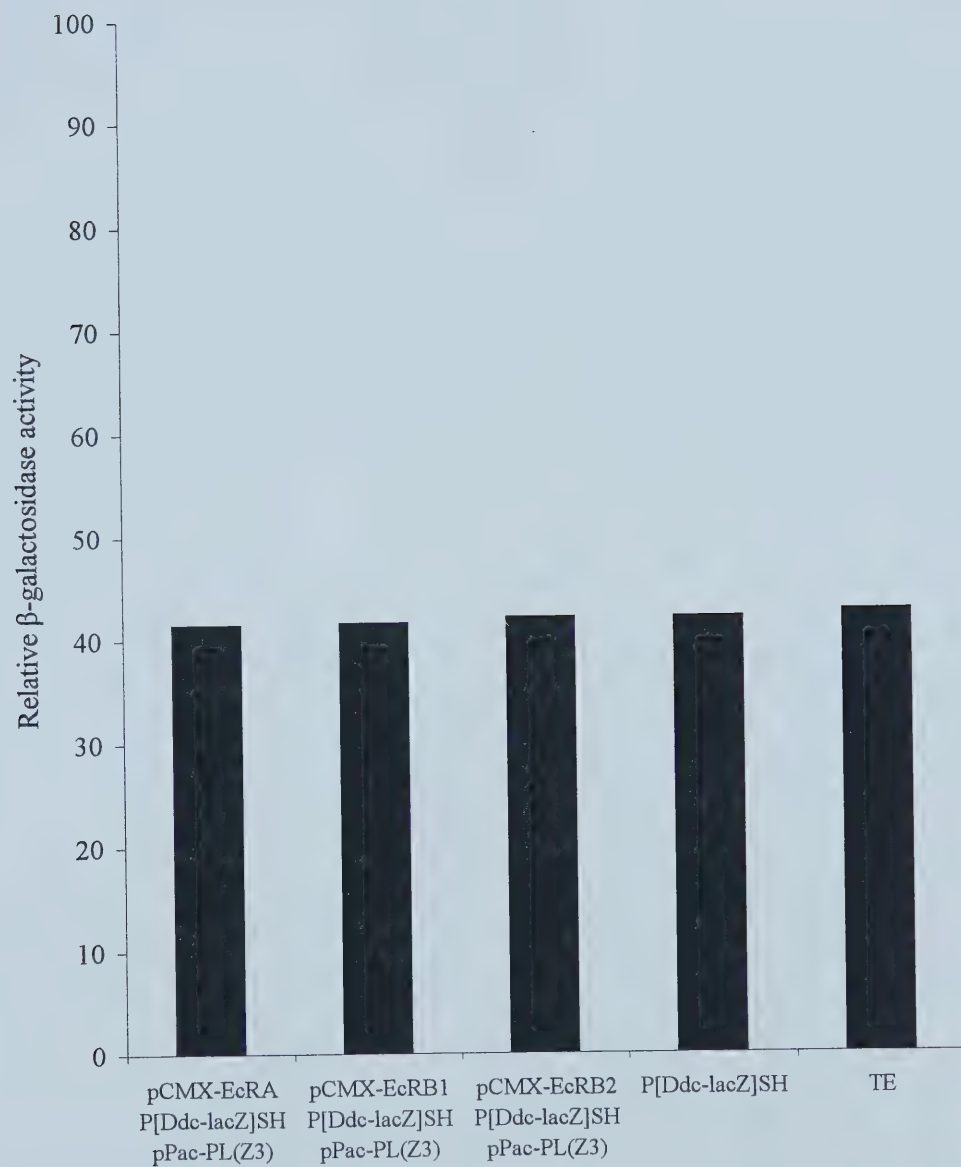
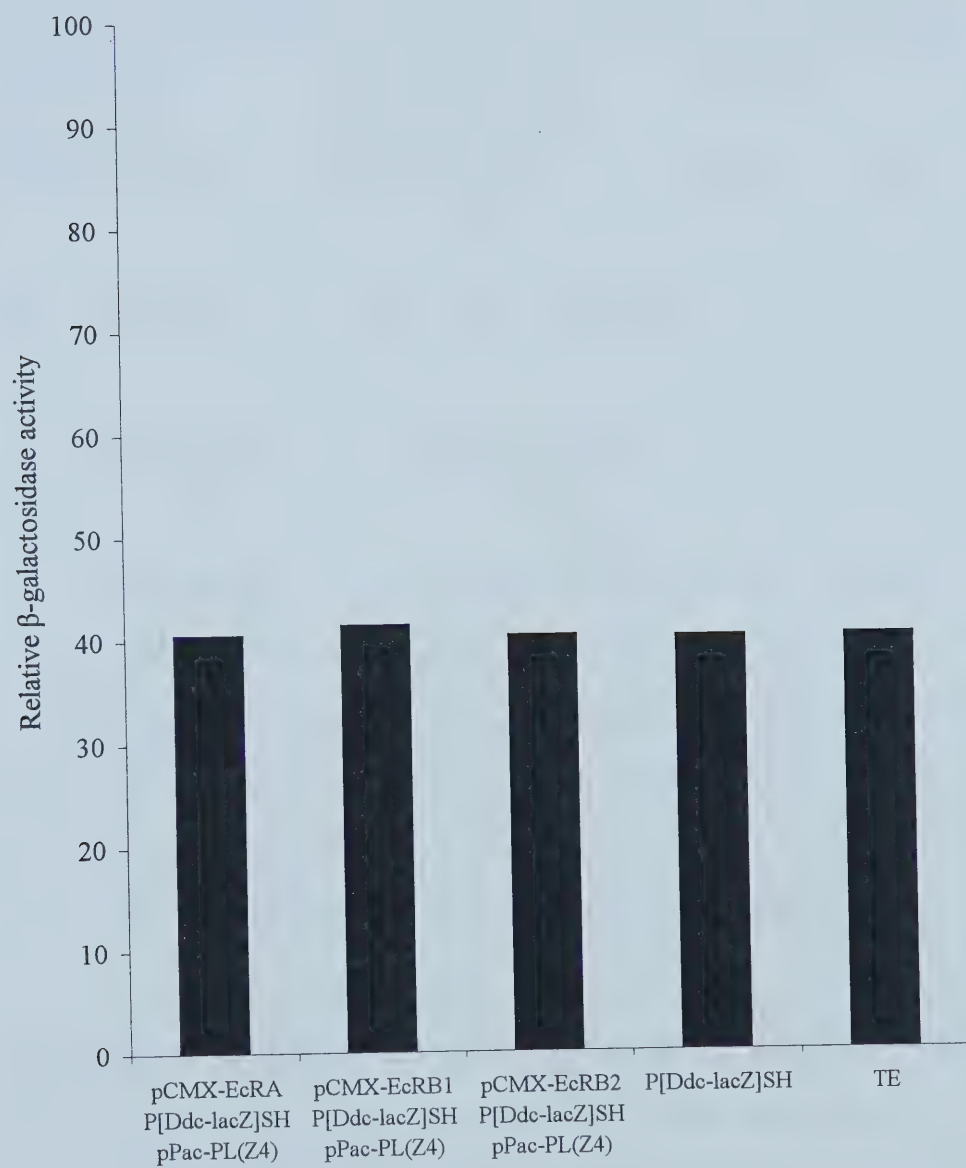


Figure III-23 The effect of exogenous BR-C Z4 protein on P[*Ddc-lacZ*]SH-generated β -galactosidase activity in L57-3-11 cells. The cells were transfected with plasmid DNAs and aliquots were either treated with ecdysone or ethanol. Cell extracts were then assayed for β -galactosidase activity. The results are shown as a ratio of the β -galactosidase activity in ecdysone treated cells compared to ethanol treated cells, with the plasmid DNAs used for each transfection being indicated on the abscissa. The values shown are the average of two separate transfection experiments, with the cell extracts from each transfection being assayed in triplicate.



Discussion

Today, much of the research in the area of *Drosophila* metamorphosis is focused on determining how a single signal, namely an increased titre of ecdysone, is converted into the divergent developmental pathways that make up metamorphosis. The available evidence indicates that *EcR* is one of the genes responsible for generating the tissue-specificity of the responses to ecdysone at pupariation, which represents the onset of metamorphosis. Specifically, Talbot *et al.* (1993) have shown that the expression of EcR-A and EcR-B1 proteins at pupariation is well correlated with the fate of that tissue at this stage in development. Furthermore, the epidermis, which is the site of the majority of DDC expression, was shown to predominantly express EcR-B1. Lastly, Bender *et al.* (1997) demonstrated that the EcR-B1 isoform is redundant until the onset of metamorphosis, when it then becomes essential for continued viability of the organism. These results suggest that EcR-B1 may mediate the ecdysone-dependent *Ddc* expression seen at pupariation. They also form the foundation for this thesis, which is an investigation into the role of the *EcR* gene in mediating the response of the *Ddc* gene to the prepupal pulse of ecdysone.

The paradigm that directed our thinking descends from an interest in the hormonal control of insect metamorphosis. Originally, basic anatomical experiments were able to show that the ring gland generated substances that were essential to initiate the process of metamorphosis (Fraenkel, 1935). Later on, developments in the field of biochemistry allowed researchers to isolate this substance, now known as ecdysone

(Karlson and Sekeris, 1966). Finally, the use of cytogenetics provided Ashburner and colleagues with the evidence to create a model that has defined the genetic framework of the response to the release of ecdysone in *Drosophila* (Ashburner *et al.*, 1974; Andres and Thummel, 1992). Most recently, modern molecular biology has allowed researchers to isolate many of the genes involved in the physiological response to ecdysone (Andres and Thummel, 1992; Thummel, 1996).

The role of EcR-B1 in *Ddc* induction at pupariation

The availability of *Drosophila* stocks carrying the *EcR-B1*-specific mutations made it possible to examine *Ddc* expression in these organisms. Crosses were performed to generate *EcR-B1* mutant larvae, along with their wild-type siblings, and these organisms were collected at the late-third instar stage and processed to extract total RNA. An RT-PCR assay demonstrated that while the wild-type organisms at this stage expressed very high steady-state levels of *Ddc* RNA, the mutant organisms had no measurable level of *Ddc* RNA (Fig. III-8).

To confirm these results, we examined the expression levels of the P[*Ddc-lacZ*]SH construct in *EcR-B1* mutant and wild-type backgrounds. The P[*Ddc-lacZ*]SH construct has been shown to generate an 18-fold increase in expression at the onset of metamorphosis, when compared to expression in early-wandering third instar larvae (Chen *et al.*, 2002a). When placed in an *EcR-B1* mutant background at the onset of metamorphosis, the P[*Ddc-lacZ*]SH construct generated only 28% of the activity produced by the same construct in a wild-type organism at the same stage (Fig. III-

13). These results, taken in conjunction with those of the RT-PCR assay, demonstrate that the EcR-B1 protein isoform participates in the induction of *Ddc* at pupariation.

Recently, a study done by Cherbas *et al.* (2003) has produced evidence that seemingly contradicts the conclusion that EcR-B1 participates in the expression of *Ddc* at pupariation. Initially, the authors demonstrated that the expression of a dominant-negative mutant EcR (EcR-DN) in the epidermis of third instar larvae resulted in lethality in the later portions of this stage. Subsequently, the authors co-expressed the EcR-DN with the three functional EcR isoforms on a one-by-one basis. They found that co-expression of EcR-B2, but not EcR-B1 or EcR-A, rescued the EcR-DN-induced lethality. These results suggest that EcR-B2, and not EcR-B1, may be responsible for the response to ecdysone in the epidermis of third instar larvae. However, it is important to note that the expression of both the EcR-DN and the functional EcR protein isoforms was generated by an *Eip* driver, which becomes active at the mid-third instar transition (Cherbas *et al.*, 2003). The mid-third instar transition is a point associated with a small peak of ecdysone expression (Richards, 1981; Riddiford, 1993) and the onset of expression of a number of genes that are likely important to the subsequent onset of metamorphosis (Huet *et al.*, 1993; Andres *et al.*, 1993; von Kalm *et al.*, 1994; see below for further discussion). It seems most plausible that EcR-B2 participates in events at the mid-third instar transition, with the resulting gene expression at this point being essential to the subsequent onset of metamorphosis. Most likely, the EcR-B1 protein then participates in generating

specific outcomes, such as producing induction of *Ddc*, that occur during metamorphosis.

EcR-B1 and the silencing element(s) of the *Ddc* promoter

Chen *et al.* (2002a) have produced evidence indicating that a silencing element(s) lies in the -2067 to -1427 bp segment of the 5'-flanking region of the *Ddc* gene relative to the transcription start site. The authors found that *Ddc*-driven reporter constructs lacking this portion of the *Ddc* 5'-flanking region generated precocious expression during the early-wandering phase of the third instar stage. Preliminary results suggest that binding of the DHR38 protein to this region of DNA is responsible for the repression (Hodgetts, unpublished). To determine if EcR-B1 plays a role in this precocious expression, we created organisms with the aforementioned reporter constructs in an *EcR-B1* mutant background and assayed the organisms when they entered the early-wandering phase of the third instar stage. The results show a complete loss of precocious expression in the *EcR-B1* mutant organisms (Fig. III-12), suggesting that EcR-B1 participates in generating this precocious expression. Paradoxically, Chen *et al.* (2002a) have shown that eliminating the functional EcRE fails to eliminate the precocious expression in the aforementioned constructs. However, it is important to recognize that a functional EcR-B1 protein is still present under the experimental conditions of Chen *et al.* (2002a). When considered in light of this fact, the combined results suggest that the EcR-B1 protein generates this precocious expression in an indirect manner.

If this precocious expression is indirectly dependent upon a functional EcR-B1 protein, it becomes important to define the nature of this link between the precocious expression and the receptor molecule. It is tempting to suggest that the *BR-C* gene fills this role because it is induced by ecdysone (Belyaeva *et al.*, 1980; Karim *et al.*, 1993) and its protein products participate in the induction of the *Ddc* gene at pupariation (Hodgetts *et al.*, 1995; Chen *et al.* 2002b). However, this is unlikely the case as precocious expression is undiminished by the removal of the *cis*-acting sequence of the *Ddc* gene that is responsible for conferring ecdysone sensitivity upon the gene (Chen *et al.*, 2002a; Chen *et al.*, 2002b).

At this point, a more plausible explanation for this precocious expression is that it is the result of non-specific transcription of the gene. P[*Ddc-lacZ*]RH, a reporter construct with only a small segment of the upstream region of *Ddc*, also exhibits this precocious expression (Chen *et al.*; 2002a; this study). This promoter fragment, extending from -382 to +174 bp with respect to the transcription start site, seems to possess little in the way of important regulatory sequence beyond the functional EcRE that is wholly unnecessary for this precocious expression (Chen *et al.*; 2002a). Also, the precocious expression occurs at a time when there is a high degree of transcriptional activity in the organism (Huet *et al.*, 1993; Andres *et al.*, 1993; von Kalm *et al.*, 1994). A growing body of evidence suggests that gene expression is often controlled, at least in part, by chromatin remodeling, which is itself dependent upon modifications that include DNA methylation, histone acetylation, and histone methylation (Geiman and Robertson, 2002; Grunstein, 1997; Wolffe *et al.*, 1997).

Furthermore, studies of vertebrate nuclear receptors with homology to EcR suggest that these receptors depend upon protein complexes with histone acetyltransferase activity to generate gene expression (reviewed in Torchia *et al.*, 1998 and Moras and Gronemeyer, 1998). The aforementioned points suggest that chromatin remodeling is sufficiently widespread to cause precocious expression and that the EcR-B1 protein plays an essential role in generating this chromatin remodeling in wandering-third instar larvae. Under this scenario, the repressor would be necessary to prevent aberrant, and likely lethal, *Ddc* gene expression in wandering-third instar larvae. However, this precocious expression is still poorly understood and will require more systematic study before any firm conclusions can be drawn.

Analysis of *Ddc* expression in a cell-culture system

A viable cell-culture system has the potential to be of great benefit when studying the structure and function of the *Ddc* gene. It would enable the experimenter to rapidly assay the functional effects of any changes he has made to the *Ddc* gene.

Furthermore, by using the ecdysone receptor-deficient L57-3-11 cell line, it is possible for the experimenter to both control for and study the regulatory effects of the three EcR isoforms. The experimenter can do this by reintroducing only the appropriate isoform or isoforms into the cell-culture system prior to induction with ecdysone.

Our initial experiment, which consisted of transfecting L-57-3-11 cells with the P[*Ddc-lacZ*]SH and the pCMX-EcRB1 plasmids (see Table II-1, Fig. III-9), failed to

generate measurable LacZ expression (Fig. III–14). Similar experiments, which consisted of transfecting the L-57-3-11 cells with the P[*Ddc-lacZ*]SH plasmid and either the pCMX-EcRA plasmid or pCMX-EcRB2 plasmid, also failed to generate measurable LacZ activity (Fig. III–14). The aforementioned experiments were then repeated with the P[*Ddc-lacZ*]BH plasmid and then the P[*Ddc-lacZ*]RH plasmid in place of P[*Ddc-lacZ*]SH plasmid. These combinations of reporter vectors and EcR expression vectors also failed to generate any measurable LacZ activity (Figs. III–15, III–16).

At this point, we thought it possible that the absence of the necessary BR-C Z2 protein (Hodgetts *et al.*, 1995) might have been responsible for the lack of measurable LacZ activity. To test this hypothesis, we transfected L-57-3-11 cells with pPac-PL(Z2) (see Results) and all possible pair wise combinations of P[*Ddc-lacZ*]SH, P[*Ddc-lacZ*]BH, or P[*Ddc-lacZ*]RH and pCMX-EcRA, pCMX-EcRB1, or pCMX-EcRB2. Unfortunately, the presence of the BR-C Z2 protein was unable to generate levels of LacZ activity from the reporter plasmids that were above control values (Fig. III–20). Finally, we repeated these experiments with pPac-PL(Z1), pPac-PL(Z3), and then pPac-PL(Z4) in place of pPac-PL(Z2). This last set of experiments also failed to generate a measurable level of LacZ activity (Figs. III–21, III–22, III–23). Given this repeated inability to generate measurable LacZ activity, we concluded that the system was incapable of recapitulating the endogenous expression patterns of the *Ddc* gene. Subsequently, each of these transfection experiments was repeated by others investigators using the SH, BH, and RH fragments of the *Ddc* gene

(Fig. III-9) inserted into the pGL3-Basic (Promega) expression vector (Ross Hodgetts, pers. comm.). Because pGL3-Basic produces luciferase as its reporter molecule, it was hoped that this would eliminate the possibility that the background LacZ activity (see Results; Best-Belpomme *et al.*, 1978) was masking induced LacZ activity. However, these experiments also failed to generate a measurable level of reporter activity, which strengthens the argument that this cell-culture system is incapable of recapitulating *Ddc* expression.

Although further study of the *Ddc* gene in this cell-culture system was precluded, these negative results were not totally uninformative. We know that the L-57-3-11 cell line is derived from embryonic tissue (Cherbas and Cherbas, 1997; Andres and Cherbas, 1992) and most likely does not accurately recapitulate the environment found in cells that are nearing the onset of metamorphosis. In addition, inputs from the ecdysone-bound receptor and the BR-C Z2 protein, which largely mediate *Ddc* transcription at pupariation *in vivo* (Chen *et al.*, 2002a; Chen *et al.*, 2002b; Hodgetts *et al.*, 1995), were insufficient to generate expression from the reporter constructs used in the experiment. Overall then, this suggests that the cellular environment plays an important role in generating *Ddc* expression at pupariation.

Conclusions

It is generally believed that an event usually referred to as the mid-third instar transition is a key point in the development of *Drosophila melanogaster* (discussed in Thummel, 1996). The feeling is that the mid-third instar transition is initiated by a

small peak in the ecdysone titre around the middle of the third larval instar (Richards, 1981; Riddiford, 1993). This peak is rapidly followed by a significant change in the pattern of gene expression, including the onset of expression of the *BR-C*, *EcR*, *E74*, and Glue genes, that persists until the onset of metamorphosis (Huet *et al.*, 1993; Andres *et al.*, 1993; von Kalm *et al.*, 1994). In addition, the larvae switch from a feeding behavior to a wandering behaviour soon after the occurrence of this small peak in the ecdysone titre. A link between this peak in the ecdysone titre and the changes in the pattern of gene expression and the onset of wandering behaviour has been difficult to demonstrate because of the poor synchrony of *Drosophila* larval development. However, the significance of this mid-third instar transition is reinforced by work with *Manduca sexta*, where the endocrinology is well defined. *Manduca sexta* exhibit a similar rise in the ecdysone titre during the final larval instar that has been shown to generate important changes in epidermal gene expression and a switch to wandering behaviour in the organism (Riddiford, 1986).

Given the aforementioned scenario, the small peak in the ecdysone titre in mid-third instar larvae seems to be responsible for initiating the chain of physiological events necessary to bring about metamorphosis. Furthermore, because the *Eip*-driven expression of the *EcR*-DN described by Cherbas *et al.* (2003) begins at the mid-third instar transition, its lethal phenotype is most likely the result of the dominant negative receptor blocking ecdysone-induced gene expression seen at this point. It then follows that by showing that *EcR*-B2 is capable of rescuing this lethal phenotype,

Cherbas *et al.* (2003) demonstrated that this isoform is responsible for mediating the ecdysone-induced gene expression at the mid-third instar transition.

Another consequence of the mid-third instar transition is the widespread gene expression that occurs between the pulse of ecdysone that initiates the transition and the onset of metamorphosis (Huet *et al.*, 1993; Andres *et al.*, 1993; von Kalm *et al.*, 1994). The presence of this gene expression has two key ramifications with respect to this thesis. First, it is possible that the gene expression is due, at least in part, to chromatin remodeling. Given that the gene expression seems widespread during this period, it is possible that chromatin remodeling is sufficiently widespread to cause the precocious expression of the *Ddc* gene described by Chen *et al.* (2002a). This is in keeping with the possible mechanism for this precocious expression discussed above. Second, the widespread gene expression seen following the mid-third instar transition indicates that a large number of gene products are likely necessary to bring about the metamorphic process. This evidence supports the finding in the cell-culture system employed in this thesis, which suggest that inputs from ecdysone and BR-C alone are insufficient to generate high levels of *Ddc* expression.

Finally, the onset of EcR expression at the mid-third instar transition (Huet *et al.*, 1993; Andres *et al.*, 1993) raises the possibility that the peak in the ecdysone titre at this point is ultimately responsible for generating the complementary expression patterns of EcR-B1 and EcR-A seen in late-third instar larvae by Talbot *et al.* (1993). This becomes especially important in light of our results, which show that ecdysone-

induced *Ddc* expression at pupariation, which largely occurs in the epidermis, is specifically mediated by the EcR-B1 protein isoform, which is the predominant isoform in the epidermis at this time (Talbot *et al.*, 1993). These results support the general view that the three EcR isoforms should differ in their abilities to activate the expression of particular genes. Furthermore, they are supported by the recent work of Hu *et al.* (2003), which demonstrated that either EcR-B1 or EcR-B2, but not EcR-A, is capable of generating transcriptional activation of a semisynthetic *Eip71CD* promoter in *Drosophila* Kc cells. Viewed in a broader sense, these results also show a link between the expression of an EcR isoform in a tissue and a developmental outcome. Specifically, this thesis has shown a link between EcR-B1 expression in the epidermis and the tanning of the larval cuticle, a developmental event known to be dependent upon the presence of DDC in the epidermis. Overall then, these results support the link between the expression of a specific EcR isoform and the fate of a tissue that was originally described by Talbot *et al.* (1993). However, these results also raise the question of how exactly this complementary pattern of EcR isoform expression is generated in the first place. It is this question that makes the mid-third instar transition relevant to these results. The *EcR* transcription that begins at this point and continues until the onset of metamorphosis is likely responsible for generating the pattern of EcR-A and EcR-B1 expression shown by Talbot *et al.* (1993). Yet it remains to be shown exactly which *EcR* transcripts are being generated during this period. Furthermore, almost next to nothing is known about the factors that cause each given tissue to express either EcR-A or EcR-B1. The answers to these unknowns will go towards explaining the relevance of the results in this thesis

and the likely starting point in the search for these answers is the mid-third instar transition.

Bibliography

- Akimaru H, Chen Y, Dai P, Hou DX, Nonaka M, Smolik SM, Armstrong S, Goodman RH, Ishii S (1997). *Drosophila* CBP is a co-activator of cubitus interruptus in hedgehog signalling. *Nature* **386**:735-738.
- Andres AJ, Thummel CS (1994). Methods for quantitative analysis of transcription in larvae and prepupae. *Methods Cell Biol.* **44**: 565-573.
- Andres AJ, Thummel CS (1992). Hormones, puffs and flies: the molecular control of metamorphosis by ecdysone. *Trends Genet.* **8**:132-138.
- Andres AJ, Fletcher JC, Karim FD, Thummel CS (1993). Molecular analysis of the initiation of insect metamorphosis: A comparative study of *Drosophila* ecdysteroid-regulated transcription. *Dev. Biol.* **160**: 388-404.
- Andres AJ, Cherbas P (1992). Tissue-specific ecdysone responses: regulation of the *Drosophila* genes *Eip38/29* and *Eip40* during larval development. *Development* **116**: 865-876.
- Ashburner M, Richards G (1976). Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. III. Consequences of ecdysone withdrawal. *Dev. Biol.* **54**: 241-255.
- Ashburner M, Chihara C, Meltzer P, Richards G (1974). Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbour Symp. Quant. Biol.* **38**: 655-662.
- Ausubel FM, Brent R, Kingston RE, Moore DO, Seidman JG, Smith JA, Struhl K (1994). Current Protocols in molecular biology. Green publishing associates, Inc. and John Wiley and Sons, Inc., New York City, NY.
- Bai J, Uehara Y, Montell DJ (2000). Regulation of invasive cell behavior by TAIMAN, a *Drosophila* protein related to AIB1, a steroid receptor coactivator amplified in breast cancer. *Cell* **103**: 1047-1058.
- Bayer CA, vonKalm L, Fristrom JW (1997). Relationships between protein isoforms and genetic functions demonstrate functional redundancy at the *Broad-Complex* during *Drosophila* metamorphosis. *Dev. Biol.* **187**: 267-282.
- Bayer CA, Holley B, Fristrom JW (1996). A switch in *Broad-Complex* zinc-finger isoform expression is regulated posttranscriptionally during metamorphosis of *Drosophila* imaginal discs. *Dev. Biol.* **177**: 1-14.

- Beckstead R, Ortiz JA, Sanchez C, Prokopenko SN, Chambon P, Losson R, Bellen HJ (2001). Bonus, a *Drosophila* Homolog of TIF1 Proteins, Interacts with Nuclear Receptors and Can Inhibit β FTZ-F1-Dependent Transcription. *Molecular Cell* **7**: 753–765.
- Belyaeva ES, Aizenzon MG, Semeshin VF, Kiss II, Koczka K, Baritcheva EM, Gorelova TD, Zhimulev IF (1980). Cytogenetic analysis of the 2B3-4–2B11 region of the X-chromosome of *Drosophila melanogaster*. I. Cytology of the region and mutant complementation groups. *Chromosoma* **81**: 281–306.
- Beermann, W (1961). Ein Balbiani-ring als Locus einer Speicheldrüsen-Mutation. *Chromosoma* **12**: 1–25.
- Bender M, Imam FB, Talbot WS, Ganetzky B, Hogness DS (1997). *Drosophila* ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell* **91**: 777–788.
- Best-Belpomme M, Courgeon AM, Rambach A (1978). β -Galactosidase is induced by hormone in *Drosophila melanogaster* cell cultures. *Proc. Natl. Acad. Sci. USA*. **75**: 6102–6106.
- Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, Carlquist M (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389**: 753–758
- Burtis KC, Thummel CS, Jones CW, Karim FD, Hogness DS (1990). The *Drosophila* 74EF early puff contains *E74*, a complex ecdysone-inducible gene that encodes two *ets*-related proteins. *Cell*. **61**: 85–99.
- Chen L, Reece C, O’Keefe SL, Hawryluk GW, Engstrom MM, Hodgetts RB (2002a). Induction of the early-late *Ddc* gene during *Drosophila* metamorphosis by the ecdysone receptor. *Mech. Dev.* **114**: 95–107.
- Chen L, O’Keefe SL, Hodgetts RB (2002b). Control of *Dopa decarboxylase* gene expression by the *Broad-Complex* during metamorphosis in *Drosophila*. *Mech. Dev.* **119**: 145–156.
- Cherbas L, Hu X, Zhimulev I, Belyaeva E, Cherbas P (2003). EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. *Development* **130**: 271–284.
- Cherbas L, Cherbas P (1997). “Parahomologous” gene targeting in *Drosophila* cells: an efficient, homology-dependent pathway of illegitimate recombination near a target site. *Genetics* **145**: 349–358.

- Cherbas L, Lee K, Cherbas P (1991). Identification of ecdysone response elements by analysis of the *Drosophila* Eip 28/29 gene. *Genes Dev.* **5**: 120–131.
- Clark WC, Doctor J, Fristrom JW, Hodgetts RB (1986). Differential responses of the dopa decarboxylase gene to 20-OH ecdysone in *Drosophila melanogaster*. *Dev. Biol.* **114**: 141–150.
- DiBello PR, Withers DA, Bayer CA, Fristrom JW, Guild GM (1991). The *Drosophila Broad-Complex* encodes a family of related proteins containing zinc fingers. *Genetics* **129**: 385–397.
- Emery IF, Bedian V, Guild GM (1994). Differential expression of *Broad-Complex* transcription factors may forecast tissue-specific developmental fates during *Drosophila* metamorphosis. *Development* **120**: 3275–3287.
- Fletcher JC, Burtis KC, Hogness DS, Thummel CS (1995). The *Drosophila* E74 gene is required for metamorphosis and plays a role in the polytene chromosome puffing response to ecdysone. *Development* **121**: 1455–1465.
- Fraenkel G (1935). A hormone causing pupation in the blowfly, *Calliphora erythrocephala*. *Proc. R. Soc. Ser. B.* **118**: 1–12.
- Garen A, Kauvar L, Lepesant J-A (1977). Roles of ecdysone in *Drosophila* development. *Proc. Natl. Acad. Sci. USA.* **74**: 5099–5103.
- Geiman TM, Robertson KD (2002). Chromatin remodeling, histone modifications, and DNA methylation—how does it all fit together? *J. Cell. Biochem.* **87**: 117–125.
- Gronemeyer H, Laudet V (1995). Nuclear Receptors. *Protein Profile* **2**: 1173–1308.
- Grunstein M (1997). Histone acetylation in chromatin structure and transcription. *Nature* **389**: 349–352.
- Hill RJ, Segraves WA, Choi D, Underwood PA, Macavoy E (1993). The reaction with polytene chromosomes of antibodies raised against *Drosophila* E75A protein. *Insect Biochem. Mol. Biol.* **23**: 99–104.
- Hirsh J, Morgan BA, Scholnick SB (1986). Delimiting regulatory sequences of the *Drosophila melanogaster* Ddc gene. *Mol. Cell. Biol.* **6**: 4548–4557.
- Hirsh J, Davidson N (1981). Isolation and characterization of the dopa decarboxylase gene of *Drosophila melanogaster*. *Mol. Cell. Biol.* **1**: 475–485.
- Hodgetts RB, Clark WC, O'Keefe SL, Schouls M, Crossgrove K, Guild, GM, von Kalm L (1995). Hormonal induction of dopa decarboxylase in the epidermis

- of *Drosophila* is mediated by the *Broad-Complex*. *Development* **121**: 3913–3922.
- Hu X, Cherbas L, Cherbas P (2003). Transcription activation by the ecdysone receptor (EcR/USP): identification of activation functions. *Mol. Endocrinol.* **17**: 716–731.
- Huet F, Ruiz C, Richards G (1993). Puffs and PCR: the *in vivo* dynamics of early gene expression during ecdysone responses in *Drosophila*. *Development* **118**: 613–627.
- Karim FD, Guild GM, Thummel CS (1993). The *Drosophila Broad-Complex* plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis. *Development* **118**: 977–988.
- Karim FD, Thummel CS (1992). Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. *EMBO J.* **11**: 4083–4093.
- Karlson P, Sekeris CE (1966). Ecdysone, an insect steroid hormone, and its mode of action. *Recent Prog. Horm. Res.* **22**: 473–502.
- Kiss I, Beaton AH, Tardiff J, Fristrom D, Fristrom JW (1988). Interactions and developmental effects of mutations in the *Broad-Complex* of *Drosophila melanogaster*. *Genetics* **118**: 247–259.
- Koelle MR, Talbot WS, Segraves WA, Bender MT, Cherbas P, Hogness DS (1991). The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67**: 59–77.
- Konrad KD, Marsh JL (1987). Developmental expression and spatial distribution of *dopa* decarboxylase in *Drosophila*. *Dev. Biol.* **122**: 172–185.
- Kraminsky GP, Clark WC, Estelle MA, Gietz RD, Sage BA, O'Connor JD, Hodgetts RB (1980). Induction of translatable mRNA for *dopa* decarboxylase in *Drosophila*: An early response to ecdysterone. *Proc. Natl. Acad. Sci. USA.* **77**: 4175–4179.
- Krasnow MA, Saffman EE, Kornfeld K, Hogness DS (1989). Transcriptional activation and repression by Ultrabithorax proteins in cultured *Drosophila* cells. *Cell* **57**: 1031–1043.
- Lambert B, Daneholt, B (1975). Microanalysis of RNA from defined cellular components. *Methods Cell Biol.* **10**: 17–47.
- Lambert, B (1972). Repeated DNA sequences in a Balbiani ring. *J. Mol. Biol.* **72**: 65–75.

- Lindsley D, Zimm GG (1992). *The Genome of Drosophila melanogaster*. Academic press, San Diego, CA.
- Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schultz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM (1995). The nuclear receptor superfamily: the second decade. *Cell* **83**: 835–839.
- Mangelsdorf DJ, Evans RM (1995). The RXR heterodimers and orphan receptors. *Cell* **83**: 841–850.
- Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM (1990). Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* **345**: 224–229.
- Marsh JL, Wright TRF (1980). Developmental relationship between dopa decarboxylase, dopamine acetyltransferase, and ecdysone in *Drosophila*. *Dev. Biol.* **80**: 379–387.
- Moras D, Gronmeyer H (1998). The nuclear receptor ligand-binding domain: structure and function. *Current Opinion in Cell Biology* **10**: 384–391.
- Mugat B, Brodu V, Kejzlarova-Lepesant J, Antoniewski C, Bayer CA, Fristrom JW, Lepesant JA (2000). Dynamic expression of *Broad-Complex* isoforms mediates temporal control of an ecdysteroid target gene at the onset of *Drosophila* metamorphosis. *Dev. Biol.* **227**: 104–117.
- O’Keefe S, Schouls M, Hodgetts RB (1995). Epidermal cell-specific quantitation of *dopa decarboxylase* mRNA in *Drosophila* by competitive RT-PCR: an effect of *Broad-Complex* mutants. *Dev. Genet.* **16**: 77–84.
- Oro AE, McKeown M, Evans RM (1990). Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. *Nature* **347**: 298–301.
- Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engstrom O, Ljunggren J, Gustafsson JA, Carlquist M (1999). Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO J.* **18**:4608–4618
- Richards G (1981). The radioimmune assay of ecdysteroid titres in *Drosophila melanogaster*. *Mol. Cell. Endocrinol.* **21**: 181–197.
- Riddiford LM (1993). Hormones and *Drosophila* development. In: *The Development of Drosophila*, M Bate and A Martinez-Arias, eds. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY.

- Riddiford LM (1986). Hormonal regulation of sequential larval cuticular gene expression. *Arch. Insect Biochem. Physiol. Suppl.* **1**: 75–86.
- Riddihough G, Pelham HRB (1987). An ecdysone response element in the *Drosophila hsp27* promoter. *EMBO J.* **6**: 3729–3734.
- Scholnick SB, Bray SJ, Morgan BA, McCormick CA, Hirsh J (1986). CNS and hypoderm regulatory elements of the *Drosophila melanogaster dopa* decarboxylase gene. *Science* **234**: 998–1002.
- Scholnick SB, Morgan BA, Hirsh J (1983). The cloned *dopa decarboxylase* gene is developmentally regulated when reintegrated into the *Drosophila* genome. *Cell* **34**: 37–45.
- Schouls M (1993): Mutations in the *Broad-Complex* affect dopa decarboxylase expression at pupariation in *Drosophila melanogaster*. M.Sc. Thesis, University of Alberta (Edmonton).
- Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**: 927–937.
- Segraves WA, Hogness DS (1990). The *E75* ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. *Genes Dev.* **4**: 204–219.
- Talbot WS, Swyryd EA, Hogness DS (1993). *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* **73**: 1323–1337.
- Thomas HE, Stunnenberg HG, Stewart AF (1993). Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. *Nature* **362**: 471–475.
- Thummel CS (1996). Files on steroids—*Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet.* **12**: 306–310.
- Thummel CS, Burtis KC, Hogness DS (1990). Spatial and temporal patterns of *E74* transcription during *Drosophila* development. *Cell* **61**: 101–111.
- Thummel CS, Boulet AM, Lipshitz HD (1988). Vectors for *Drosophila* P-element-mediated transformation and tissue culture transfection. *Gene* **74**: 445–456.
- Torchia J, Glass C, Rosenfeld MG (1998). Co-activators and co-repressors in the integration of transcriptional responses. *Current Opinion in Cell Biology* **10**: 373–383.

- Tsai CC, Kao HY, Yao TP, McKeown M, Evans RM (1999). SMRTER, a *Drosophila* nuclear receptor coregulator, reveals that EcR-mediated repression is critical for development. *Molecular Cell* **4**: 175–186.
- Tzolovsky G, Deng WM, Schlitt T, Bownes M (1999). The function of the *Broad-Complex* during *Drosophila melanogaster* oogenesis. *Genetics* **153**: 1371–1381.
- Urness LD, Thummel CS (1995). Molecular analysis of a steroid-induced regulatory hierarchy: the *Drosophila* E74A protein directly regulates *L71-6* transcription. *EMBO J.* **14**: 6239–6246.
- Von Kalm L, Crossgrove K, Von Seggern D, Guild GM, Beckendorf SK (1994). The *Broad-Complex* directly controls a tissue-specific response to the steroid hormone ecdysone at the onset of *Drosophila* metamorphosis. *EMBO J.* **13**: 3505–3516.
- Wolffe AP, Wong J, Pruss D (1997). Activators and repressors: making use of chromatin to regulate transcription. *Genes Cells.* **2**: 291–302.
- Wright TRF (1987). The genetics of biogenic amine metabolism, sclerotization, and melanization of *Drosophila melanogaster*. *Adv. Genet.* **24**: 127–222.
- Wurtz J-M, Bourguet W, Renaud J-P, Vivat V, Chambon P, Moras D, Gronemeyer H (1996). A canonical structure for the ligand-binding domain of nuclear receptors. *Nat. Struct. Biol.* 1996 **3**:87–94. [Published erratum appears in *Nat. Struct. Biol.* 1996 **3**:206].
- Yamada S, Shimizu M, Yamamoto K (2003). Structure–function relationships of vitamin D including ligand recognition by the vitamin D receptor. *Medicinal Research Reviews* **23**: 89–115
- Yao TP, Forman BM, Jiang Z, Cherbas L, Chen JD, McKeown M, Cherbas P, Evans RM (1993). Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. *Nature* **366**: 476–479.
- Yao TP, Segraves WA, Oro AE, McKeown M, Evans RM (1992). *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell* **71**: 63–72.

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